MARCH-II Is a Syntaxin-6–binding Protein Involved in Endosomal Trafficking[□]

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Membrane-associated RING-CH (MARCH) is a recently identified member of the mammalian E3 ubiquitin ligase family, some members of which down-regulate the expression of immune recognition molecules. Here, we have identified MARCH-II, which is ubiquitously expressed and localized to endosomal vesicles and the plasma membrane. Immunoprecipitation and *in vitro* binding studies established that MARCH-II directly associates with syntaxin 6. Overexpression of MARCH-II resulted in redistribution of syntaxin 6 as well as some syntaxin-6–interacting soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) into the MARCH-II–positive vesicles. In addition, the retrograde transport of TGN38 and a chimeric version of furin to *trans*-Golgi network (TGN) was perturbed—without affecting the endocytic degradative and biosynthetic secretory pathways—similar to effects caused by a syntaxin 6 mutant lacking the transmembrane domain. MARCH-II overexpression markedly reduced the cell surface expression of transferrin (Tf) receptor and Tf uptake and interfered with delivery of internalized Tf to perinuclear recycling endosomes. Depletion of MARCH-II by small interfering RNA perturbed the TGN localization of syntaxin 6 and TGN38/46. MARCH-II is thus likely a regulator of trafficking between the TGN and endosomes, which is a novel function for the MARCH family.

INTRODUCTION

Vesicular traffic between different organelles requires highly regulated docking and fusion of a transport vesicle with a specific target membrane. This process is mediated by soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) localized to a specific subcellular compartment along the secretory and endocytic pathways (Jahn and Südhof, 1999; Chen and Scheller, 2001; Hay, 2001; Mayer, 2002). Only cognate pairs of SNAREs on transport vesicles (v-SNARE) and on target membranes (t-SNARE) form the SNARE complex through their conserved amphipathic helices (SNARE motifs) and draw two membranes into apposition, allowing membrane fusion to occur (Fasshauer *et al.*, 1998; Sutton *et al.*, 1998). Syntaxin 6 is a ubiquitously expressed SNARE that localizes to the *trans*-Golgi network (TGN) and endosomes (Bock *et al.*, 1996, 1997). It is comprised of an N-terminal helical domain (H1 domain) followed by a SNARE motif (H2 domain) and a C-terminal membrane anchor (Bock *et al.*, 1996; Wendler and Tooze, 2001; Misura *et al.*, 2002). Syntaxin 6 is thought to

function in multiple membrane-trafficking events because it forms a complex with a varied set of SNAREs: syntaxin 16, Vti1a/Vti1-rp2, and VAMP3/cellubrevin or VAMP4 (Xu *et al.*, 1998; Steegmaier *et al.*, 1999; Kreykenbohm *et al.*, 2002; Mallard *et al.*, 2002); syntaxin 7, Vti1b, and VAMP7 or VAMP8 (Wade *et al.*, 2001); or SNAP-29/GS32 (Wong *et al.*, 1999). Recent studies have demonstrated that syntaxin 6 plays roles in the early/recycling endosomes-to-TGN transport (Mallard *et al.*, 2002), maturation of secretory granules (Klumperman *et al.*, 1998; Wendler *et al.*, 2001; Kuliawat *et al.*, 2004), regulation of GLUT4 trafficking (Perera *et al.*, 2003; Shewan *et al.*, 2003), and neutrophil exocytosis (Martin-Martin *et al.*, 2000).

Although SNAREs are sufficient for docking and fusion of artificial membrane liposomes *in vitro* (Weber *et al.*, 1998; McNew *et al.*, 2000), it is becoming increasingly clear that a number of SNARE-binding proteins are required for regulation of the SNARE complex formation *in vivo* (Gerst, 2003). mVps45 (a Sec1p-like protein), FIG (a Golgi-localized PDZ protein), and EEA1 (a Rab5 effector protein) have been shown to associate with syntaxin 6 (Bock *et al.*, 1997; Tellam *et al.*, 1997; Simonsen *et al.*, 1999; Charest *et al.*, 2001; Mills *et al.*, 2001). EEA1 functions in tethering the early endosomal vesicles, before the SNARE complex formation for homotypic fusion, via direct interaction with syntaxin 13 (McBride *et al.*, 1999). Interaction with syntaxin 6 suggests that EEA1 is involved in tethering the vesicles derived from the TGN to the early endosome. Therefore, the identification and characterization of the binding partner of syntaxin 6 would provide novel insights into the molecular mechanisms of vesicular trafficking and fusion.

Here, we report the identification of a novel endosomal protein that binds to syntaxin 6. The role of this protein in the endosome-to-TGN transport pathway is demonstrated by overexpression and RNA interference experiments. During preparation of this manuscript, Bartee *et al.* (2004) reported identification of a human homologue as a member of novel

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Abbreviations used: ER, endoplasmic reticulum; EGFR, epidermal growth factor receptor; MARCH, membrane-associated RING-CH; PHD, plant homeodomain; SEAP, secretory alkaline phosphatase; shRNA, short-hairpin RNA; siRNA, small interfering RNA; SNARE, soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor; Tf, transferrin; TfR, Tf receptor; TGN, *trans*-Golgi network; TMR, tetramethylrhodamine; TRITC, tetramethyl rhodamine isothiocyanate.

ubiquitin ligase family related to viral immune evasion proteins and named it membrane-associated RING-CH (MARCH)-II. Therefore, in accord with this finding, we also refer to this novel syntaxin-6-binding protein as MARCH-II.

MATERIALS AND METHODS

Cloning of MARCH-II cDNA

A BLASTN search of the GenBank database with the rat Kir7.1 genomic sequence (accession no. AB053348) identified two expressed sequence tag (EST) sequences for rat MARCH-II (accession nos. AI013040 and AA899743). A full-length clone for rat MARCH-II was obtained from small intestine by reverse transcription-polymerase chain reaction (RT-PCR) with the primers S1 (5'-ACCATGGGCTCAGGCCTGAGGA-3') and A1 (5'-ATCTGACTCGGTCTGTCCACTA-3'). The polymerase chain reaction (PCR) product was subcloned into the *EcoRV* site of pBluescript II SK⁻ (Stratagene, La Jolla, CA), yielding pBS-MAR2, and then sequenced. An additional 5' sequence was determined by 5' rapid amplification of cDNA ends with rat small intestine polyadenylated RNA by using a 5'/3'-rapid amplification of cDNA ends kit (Boehringer Mannheim via Roche Diagnostics, Indianapolis, IN) with the antisense primers A1, A2 (5'-GGAAGACAGCCATTCTCCAGCA-3'), and A3 (5'-OTCCTCAGGCCTGAGGCCCATGGT-3'). A full-length clone for human MARCH-II was obtained from HeLa cells by RT-PCR with the primers S301 (5'-CTCCTGAACCATGGGCTCAGGCCCTGAG-3') and A1560 (5'-ACGTCCAGCCAGGGCTCTTTTATTATTC-3'). The PCR product was subcloned into the *EcoRV* site of pZerO-2 (Invitrogen, Carlsbad, CA), yielding pZerO-hMAR2. The nucleotide sequences of rat and human MARCH-II are available from the GenBank/European Molecular Biology Laboratory/DNA DataBank of Japan database under accession no. AB048838 and AB197929, respectively.

Expression Constructs

A mammalian expression vector for rat or human MARCH-II was constructed by cloning the *XhoI-EcoRI* insert from pBS-MAR2 or pZerO-hMAR2 into the same sites of pcDNA3 (Invitrogen). The furin expression vector was kindly provided by Dr. Kazuhisa Nakayama (Hatsuzawa *et al.*, 1990). All other cDNA fragments were obtained by PCR amplification and confirmed for their sequences. Green fluorescent protein (GFP)-MAR2 was constructed by cloning the cDNA fragment encoding residues 2–246 of rat MARCH-II into pEGFP-C2 (BD Biosciences Clontech, Palo Alto, CA). GFP-MAR2-244K was generated by PCR-based site-directed mutagenesis. Myc-syn6cyto was constructed by cloning the fragment encoding residues 2–233 of syntaxin 6 into pCMV-Myc (BD Biosciences Clontech). To generate FLAG-furin, the region encoding residues 710–793 of rat furin was inserted into the *EcoRI-BamHI* sites of p3 × FLAGCMV-10 (Sigma-Aldrich, St. Louis, MO). By using this plasmid as a template, a region of FLAG-furin was amplified with the primers 5'-TTGGTACCGTCAGAATTAACACCGACTAC-3' and 5'-AAGGATCCTCATCAAGGGCGCTCTGGTC-3' and inserted into the *KpnI-BamHI* sites of pSecTag (Invitrogen). Other mammalian expression vectors were constructed by cloning individual coding regions into pcDNA3, p3 × FLAGCMV-10, or p3 × FLAGCMV-14 (Sigma-Aldrich). Prokaryote expression vectors for GST-MAR2_N and GST-MAR2_C were constructed by cloning the fragment encoding residues 2–141 and 199–246 of rat MARCH-II, respectively, into pGEX-4T (Amersham Biosciences, Piscataway, NJ). His-MAR2_C was constructed by cloning the fragment encoding residues 197–246 of rat MARCH-II into pRSET (Invitrogen). Glutathione S-transferase (GST)-syn6, GST-syn6N, GST-syn6C, and GST-VAMP3 were constructed by cloning the fragments encoding residues 2–233, 2–136, and 137–233 of syntaxin 6, and residues 1–80 of VAMP3 into pGEX-4T, respectively. GST-Veli was constructed by cloning a coding region of rat Veli3 into pGEX-4.

Northern Blot Analysis

Rat multiple-tissue Northern blots (OriGene Technologies, Rockville, MD) were hybridized with a ³²P-labeled *EcoRI-HindIII* fragment of pBS-MAR2 as described previously (Mistry *et al.*, 2001).

Subcellular Fractionation

All operations were carried out at 4°C, and all solutions and buffers were added with protease inhibitors (10 mM leupeptin, 1 mM pepstatin, 5 mg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride) just before use. The membranes were lysed in HT solution (Hanks' balanced salt solution [HBSS] containing 1% Triton X-100).

Plasma Membranes. The liver (3 g) was homogenized in 30 ml of PH buffer (10 mM KCl and 10 mM HEPES-KOH, pH 7.5) containing 0.25 M sucrose. The homogenate (H1) was centrifuged for 10 min at 280 × g, and the supernatant was centrifuged for 10 min at 10,000 × g. The pellet was suspended in 15 ml of PH containing 0.25 M sucrose followed by centrifugation for 20 min at 10,000 × g. The resulting pellet was resuspended in 7 ml of PH containing 2.0

M sucrose and applied to a discontinuous sucrose gradient comprised of PH containing 1.2 and 0.25 M sucrose, which was then centrifuged for 2 h at 25,000 rpm in a Beckman SW28 rotor (Beckman Coulter, Fullerton, CA). The band at the 0.25/1.2 M sucrose interface was sedimented by centrifugation at 10,000 × g in PH containing 0.25 M sucrose.

Lysosomal Membranes. Rat liver lysosomal membranes were isolated as described previously (Ohsumi *et al.*, 1983).

Microsomal Membranes. The liver was homogenized in SPH buffer (0.25 M sucrose, 0.2 M KCl, and 10 mM HEPES-KOH, pH 7.0). The postmitochondrial supernatant (PMS) was applied to a discontinuous sucrose gradient comprising PH containing 2.0, 1.3, and 0.86 M sucrose, which was centrifuged for 90 min at 25,000 rpm in an SW28 rotor. The bands LM (PMS/0.86 M sucrose interface) and HM (0.86/1.3 M interface) were diluted threefold with 0.25 M sucrose, layered onto the 0.8 and 1.3 M sucrose cushion, respectively, and then centrifuged at 40,000 rpm for 30 min in a Beckman SW41Ti rotor. Each interface band was diluted at least fivefold with HBSS and sedimented by centrifuging at 40,000 rpm on a Beckman 80Ti rotor.

Golgi-enriched Membranes. The homogenate H1 was centrifuged twice for 20 min at 10,000 × g. The supernatant was layered onto PH containing 0.80 M sucrose followed by centrifugation for 90 min at 25,000 rpm on an SW28 rotor. The band at the sucrose interface was collected and sedimented.

Antibodies

Anti-peptide antisera (anti-MAR2_C#41 and anti-MAR2_N#51) were made in rabbits against synthetic peptides corresponding to residues 212–230 and to 42–61 of rat MARCH-II, respectively. A rabbit polyclonal anti-MAR2_N#384 was raised against a GST-N fusion protein. Antibodies were affinity purified with HiTrap NHS-activated HP (Amersham Biosciences) coupled with GST-MAR2_C (for anti-MAR2_C#41) or GST-MAR2_N (for anti-MAR2_N#51 and anti-MAR2_N#384). The following primary antibodies were purchased: polyclonal: lamp-2, VAMP3, calreticulin, and furin were from Affinity Bioreagents (Cambridge, United Kingdom); β1,4-galactosyltransferase 1 was from Santa Cruz Biotechnology (Santa Cruz, CA); syntaxin 8, syntaxin 16, and VAMP4 were from Synaptic Systems (Göttingen, Germany); and Rab6 was from Calbiochem (San Diego, CA); and monoclonal: Na⁺K⁺-ATPase α1 subunit was from Upstate Biotechnology (Lake Placid, NY); transferrin receptor (TfR) was from Zymed Laboratories (South San Francisco, CA); syntaxin 13 was from StressGen Biotechnologies (Victoria, BC, Canada); FLAG-tag (anti-FLAG M2), and epidermal growth factor (EGF) receptor (EGFR) were from Sigma-Aldrich; and the others were from BD Transduction Laboratories (Lexington, KY).

GST Pull-Down Assay

To analyze the interaction with syntaxin 6, GST fusion proteins (0.1 nmol each) and His-MAR2_C (0.25 nmol) were incubated in 1 ml of HBSS containing 0.03% Triton X-100 at 4°C overnight. Mixtures were immobilized with 40 μl of glutathione-Sepharose 4B beads (Amersham Biosciences). Beads were washed six times with 1 ml of HBSS containing 0.03% Triton X-100. The bound proteins were eluted with 60 μl of 10 mM glutathione, 50 mM Tris-HCl, pH 8.0, and 0.03% Triton X-100. To analyze the interaction with the PDZ domain, GST fusion proteins (0.5 nmol each) are incubated with either His-MAR2_C (~1 nmol) or His-MAR2_C-244K (~1 nmol) in 1 ml of 0.15 M NaCl solution containing 0.1% Triton X-100 (solution A) for 1 h at room temperature. Mixtures were added with 300 μl of glutathione-Sepharose 4B beads and further incubated for 1 h. Beads were packed in a Bio-Rad Econocolumn (0.7 × 10 cm; Bio-Rad, Hercules, CA), washed with 15 ml of solution A, and eluted with 550 μl of 10 mM glutathione, 50 mM Tris-HCl, pH 8.0, and 0.1% Triton X-100.

Affinity Chromatography Assay

Purified His-MAR2_C (1 mg) was bound to 500 μl of Talon metal affinity resins (BD Biosciences Clontech). The high-speed supernatant (800 μl) of the Triton X-100-extracted Golgi-enriched membranes was incubated with the resins overnight at 4°C. The resins were packed in a disposal Bio-Rad column (1.2-ml bed volume), washed with 10 bed volumes of HT solution, and eluted with 500 μl of 50 mM NaH₂PO₄, pH 7.0, 300 mM NaCl, and 150 mM imidazole.

Cell Culture, Transfection, and Immunoprecipitation

COS7 and HeLa cells were cultured in DMEM, and Chinese hamster ovary (CHO) cells in Ham's F-12. All media were supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin. Transfection of plasmid vector was performed using LipofectAMINE 2000 (Invitrogen). To generate a stable cell line expressing TGN38 (HeLa-TGN38), HeLa cells were transfected with the *PvuI*-linearized pcDNA3 containing TGN38 and selected with 500 μg/ml Geneticin (Sigma-Aldrich). For immunoprecipitation, cells were lysed in HT solution for 2 h at 4°C and centrifuged at 15,000 × g. The supernatants were incubated with anti-FLAG M2 agarose

(Sigma-Aldrich). After washing with HT solution, the agarose was eluted with 0.1 M glycine-HCl, pH 3.5, containing 1% Triton X-100.

Fluorescence Microscopy

Before experiments, COS7 cells expressing MARCH-II or GFP-MAR2 were incubated with 0.1 mg/ml cycloheximide (Wako Pure Chemicals, Osaka, Japan) in DMEM for 60 min at 37°C to chase nascent proteins out of the endoplasmic reticulum (ER) and Golgi complex. Cells on coverslips were fixed with 3.7% paraformaldehyde for 10 min, permeabilized with 0.1% Triton X-100 in HBSS for 10 min, and blocked with 1% FBS in HBSS for 30 min at room temperature. They were incubated with primary antibodies (diluted 1:500 each with exception of anti-MAR2_N#51 antiserum [1:200], anti-syntaxin 6 [1:200 for HeLa cells; 1:80 for COS7 cells], anti-β1,4-galactosyltransferase [1:300 for HeLa cells; 1:100 for COS7 cells], and anti-FLAG [1.2 μg/ml]) for 1 h at room temperature followed by appropriated secondary antibodies conjugated with Alexa 350, 488, or 546 (1:2000; Molecular Probes, Eugene, OR) or tetramethyl rhodamine isothiocyanate (TRITC) (1:400; Sigma-Aldrich) for 30 min. Signals were captured with an Axioskop microscope (Carl Zeiss, Thornwood, NY) equipped with an ORCA-ER charge-coupled device (CCD) camera (Hamamatsu, Bridgewater, NJ), a CSU10 confocal scanner unit (Yokogawa Electric, Tokyo, Japan), and an argon/krypton ion laser. Images were analyzed with IP Lab software (Scanalytics, Fairfax, VA). In Figures 5–10 (with the exception of Figure 7, A and G), signals were captured with an Olympus IX70 inverted microscope (Olympus, Tokyo, Japan) with a SenSys CCD camera (Photometrics, Huntington Beach, CA). Images were analyzed with MetaVue software (Universal Imaging, Downingtown, PA).

Endocytosis of Antibodies and Fluorescent Markers

Cells were incubated in DMEM with either anti-TGN38 (1 μg/ml), anti-FLAG (3.5 μg/ml), tetramethylrhodamine (TMR)- or Alexa 488-transferrin (Tf) (25 μg/ml; Molecular Probes), or TMR-epidermal growth factor (1 μg/ml; Molecular Probes) at 37°C for a given period (40 min in antibody uptake assay). To allow the endocytosed materials chase, cells were quickly washed twice and incubated in DMEM with 100 μg/ml cycloheximide for 1 h (for antibodies) or for 2 h (for TMR-epidermal growth factor) at 37°C. Cells were subsequently subjected to fluorescence microscopy.

RNA Interference

To generate a short-hairpin RNA (shRNA) expression vector targeted to human MARCH-II, the following pair of oligonucleotides with hairpin, terminator, and overhanging sequences were annealed: 5'-GATCCGTGGCTTTCCTCATCTCAACTCAAGAGAGTTAGATGAGGAAAGCCACTTTTGTGAAA-3' and 5'-AGCTTTTCCAAAAAAGTGGCTTTCCTCATCTCAACTCTCTGAAGTTAGATGAGGAAAGCCACG-3'. The resulting cDNA fragment was inserted into the *Bam*HI-*Hind*III sites of pSilencer2.1-U6 Puro vector (Ambion, Austin, TX), which was used to stably transfect HeLa-TGN38 cells. Single clones were established by puromycin selection (at 5 μg/ml concentration) for 10–14 d. All experiments were performed between passages 3 and 15 after isolation.

Other Methods

Cell surface biotinylation (Nakamura *et al.*, 1999) and assay of secretory alkaline phosphatase (SEAP) activity (Nakamura *et al.*, 2000) were performed as described previously. Tf recycling and uptake assays were performed as described previously (Wiley and Cunningham, 1982; Hu *et al.*, 2002).

RESULTS

Cloning of MARCH-II

During the determination of the nucleotide sequence of the rat gene for inward rectifier K⁺ channel Kir7.1 (Nakamura *et al.*, 2000), a previously unidentified gene in vicinity to its 3'-most exon was detected. Partial cDNA sequences for this novel gene have been deposited in the GenBank EST database (accession nos. AI013040 and AA899743). Given that the relative positions of the *Kir7.1* gene and the novel gene were reminiscent of those of the adjacent genes that encode the two components of the pancreatic ATP-sensitive K⁺ channel—Kir6.2 and the sulfonylurea receptor (Inagaki *et al.*, 1995)—we hypothesized that the new gene (*MARCH-II*) may encode an accessory component of the Kir7.1 channel and initiated the cloning of its cDNA.

Determination of the nucleotide sequence of the MARCH-II cDNA isolated from rat small intestine revealed that the predicted protein comprises 246 amino acids with a calculated molecular mass of 27.2 kDa (Figure 1A). Hydro-

pathic analysis predicted that this protein contains two transmembrane spans (underlined) between the long N-terminal and short C-terminal cytoplasmic tails. A plant-homoeodomain (PHD) finger—a C₄HC₃ zinc-binding structure also called a RING-CH finger—is found in the N-terminal cytoplasmic region (shadowed) (Aasland *et al.*, 1995). A PDZ domain-binding motif is located at the very C terminus (wavy line) (Songyang *et al.*, 1997). Northern blot analysis revealed that the MARCH-II mRNA (~1.35 kb) was present in all rat tissues examined (Figure 1B), suggesting a generalized function common to all cell types.

Despite a similar hydropathic profile for MARCH-II and Kir7.1, they share no significant similarity in either sequence or tissue distribution. Moreover, immunoprecipitation and electrophysiological studies in cotransfected cells failed to provide evidence that MARCH-II is a regulator of Kir7.1 function (our unpublished data). We therefore decided to characterize MARCH-II independently of Kir7.1.

MARCH-II Is an Integral Membrane Protein Enriched in Endosomes

To characterize the MARCH-II protein, we generated polyclonal anti-MARCH-II antibodies (anti-MAR2_C#41, anti-MAR2_N#51, and anti-MAR2_N#384). Each affinity-purified antibody specifically recognized a 27-kDa protein in the membrane fraction of HeLa cells transfected with MARCH-II (Figure S1). MARCH-II could be solubilized effectively in 1% Triton X-100 but not HBSS, 2 M NaCl, or 0.1 M NaHCO₃, pH 11.5, an extraction profile similar to calnexin, an integral membrane protein (Figure 1C). The localization of MARCH-II was analyzed by subcellular fractionation with rat liver. The membrane fractions enriched in the plasma membrane (PM), light microsomes (LMs), heavy microsomes (HMs), and lysosomes (Ls) were subjected to immunoblot analysis with anti-MAR2_C#41 and antibodies against the following marker proteins: Na⁺/K⁺-ATPase (plasma membrane), Rab6 (Golgi), syntaxin 6 (TGN and early endosome), TfR (early/recycling endosomes and plasma membrane), Rab11 (recycling endosome), cation independent-mannose 6-phosphate receptor (MPR) (late endosome), and lamp-2 (lysosomes). MARCH-II was detected predominantly in the HM fraction enriched with the endosomal proteins, and to a lesser extent in the PM fraction (Figure 1D).

To confirm the endosomal localization of MARCH-II, CHO cells were processed for confocal immunofluorescence microscopy by using anti-MAR2_C#51 antiserum. Endogenous MARCH-II was detected in punctate structures throughout the cells (Figure 2A). Intense staining was often observed at the leading edge of cells (arrows). Control experiments with preimmune serum demonstrated only negligible background staining (our unpublished data). Double staining experiments showed that the staining pattern of MARCH-II differed substantially from those of syntaxin 6, EEA1, γ-adaptin, and TfR (Figure 2B). However, higher magnification images revealed some degree of overlap in peripheral puncta (Figure 2C). These results suggest that MARCH-II is present in a subdomain of endosomes.

MARCH-II Interacts with Syntaxin 6

We next searched for proteins that bind to MARCH-II by affinity chromatography immobilized on the His₆-tagged C-terminal tail of MARCH-II (residues 197–246; His-MAR2_C). The resins were incubated with the Triton extracts of Golgi-rich membranes from rat liver. After washing, bound proteins were eluted and detected by immunoblot analysis. His-MAR2_C pulled down syntaxin 6, syntaxin 8,

syntactin 13, VAMP3, and VAMP4 (Figure 3A). Because the ratio of the band intensity of syntactin 6 in the eluate relative to that in the input was the highest, we considered syntactin 6 as a strong candidate for a MARCH-II binding protein. The complex of MARCH-II and 3 $\times$ FLAG-tagged syntactin 6 (FLAG-syn6) was immunoprecipitated with anti-FLAG beads from the Triton extracts of COS7 cells (Figure 3B), again confirming their physical interaction. To determine whether the interaction is direct or not, we performed GST pull-down assay. His-MAR2_C was incubated with a soluble recombinant GST fusion proteins containing either various portions of syntactin 6 or the entire cytosolic domain of VAMP3 as a control (Figure 3C). Figure 3D shows that His-MAR2_C bound to GST-syn6 (lane 3) as well as GST-syn6C (lane 5) and also to GST-syn6N, although with much less affinity (lane 4). There was no interaction with control GST or GST-VAMP3 (lanes 2 and 6). In conclusion, MARCH-II directly and specifically interacts with syntactin 6 through its C-terminal tail.

MARCH-II Overexpression Affects the Localization of Syntactin 6 and Syntactin-6-binding SNAREs

To gain insight into the function of MARCH-II, we analyzed the effects of its overexpression. COS7 cells were used for immunofluorescence analysis because they stably maintained their spread-flattened shape with overexpression of MARCH-II; other cells (CHO, normal rat kidney, 293T, HeLa, and 3T3-L1 cells) tended to become spherical and detach from dishes. When expressed in COS7 cells, MARCH-II (Figure 4, A–C) and N-terminally GFP-tagged MARCH-II (GFP-MAR2) (Figure 4D) exhibited scattered vesicular distribution at relatively moderate levels of ex-

pression. Although not clearly evident from these images, weak fluorescence also was detected at the plasma membrane. Importantly, syntactin 6 was dispersed to peripheral puncta from its characteristic perinuclear localization and significantly overlapped with MARCH-II (Figure 4A). On the other hand, there was less of a change in the overall distribution of EEA1 (Figure 4B) and γ -adaptin (Figure 4C). Similar results were observed in CHO cells (Figure S2). The morphology of the TGN seemed to remain intact under these conditions, because the distribution of a *trans*-Golgi/TGN marker β 1,4-galactosyltransferase was unchanged (Figure 4D).

Because syntactin 6 forms a SNARE complex with the TGN/endosome SNAREs Vti1a, VAMP3, and VAMP4 (Xu *et al.*, 1998; Steegmaier *et al.*, 1999; Kreykenbohm *et al.*, 2002; Mallard *et al.*, 2002), we further investigated the effects of MARCH-II expression on the localization of these syntactin-6-binding SNAREs. A similar relocation was observed for endogenous Vti1a (Figure 5A) and 3 $\times$ FLAG-tagged VAMP3 (FLAG-VAMP3) (Figure 5B). However, FLAG-VAMP4 did partially colocalize with GFP-MAR2 in some peripheral puncta, but its perinuclear distribution was not significantly affected (Figure 5C). We also examined other endosomal t-SNAREs, i.e., syntactin 13 (Prekeris *et al.*, 1998) and syntactin 8 (Prekeris *et al.*, 1999), neither of which has been shown to interact with syntactin 6. Expectedly, there was less change in the distribution of FLAG-syn13 (our unpublished data) and endogenous syntactin 8 (Figure 5D). The finding that MARCH-II expression influences the distribution of syntactin 6 and some syntactin-6 partners indicates a binding specificity between MARCH-II and syntactin 6, and perhaps functional correlation.

Figure 2. Subcellular localization of endogenous MARCH-II in CHO cells. (A) CHO cells were fixed, permeabilized, and stained with anti-MAR2_N#51 antiserum followed by Alexa 488-conjugated anti-rabbit IgG. Punctate staining was observed within the cytoplasm. Occasionally, intense staining was detected at the edge of the cell (arrows). Asterisks, position of nucleus. Bar, 10 μ m. (B) CHO cells were processed for double-labeled confocal microscopy with anti-MAR2_N#51 and a mouse monoclonal antibody against syntaxin 6 (a–a''), EEA1 (b–b''), γ -adaptin (c–c''), or TfR (d–d''). Primary antibodies were visualized with Alexa 546-conjugated anti-rabbit IgG and Alexa 488-conjugated anti-mouse IgG. The signals for endogenous MARCH-II are shown in green, and those for the endogenous marker proteins are shown in red. Arrowheads indicate the colocalization of MARCH-II with syntaxin 6. Asterisks, the position of nucleus. Bars, 10 μ m. (C) Higher magnified images of insets in B are shown. Arrows indicate examples of colocalization. Bars, 1 μ m.

Involvement of MARCH-II in the Endosome-to-TGN Transport Pathway

A previous report that syntaxin 6 participates in the early/recycling endosomes-to-TGN transport (Mallard *et al.*, 2002)

led us to speculate a possible role for MARCH-II in this transport step. We tested whether MARCH-II expression has effects on the localization and trafficking of TGN38, a TGN protein cycling between the TGN and plasma mem-

Figure 3. MARCH-II interacts with syntaxin 6. (A) Triton extracts of rat liver Golgi-rich membranes were incubated with either Talon resins alone (lane 2) or the resins immobilized with His-MAR2_C (lane 3). Proteins bound to the resins were eluted and subjected to immunoblotting with antibodies against the indicated proteins. One-eighth of the amount used in the binding reaction was run in parallel (lane 1). (B) Extracts from COS7 cells expressing MARCH-II alone (lane 1) or together with FLAG-Syn6 (lane 2) were incubated with anti-FLAG M2 agarose. After extensive washing, the eluates were subjected to immunoblotting with anti-FLAG M2 (top) or anti-MAR2_N#384 (middle). Extracts (2% of input) were immunoblotted with anti-MAR2_N#384 to confirm expression of MARCH-II (bottom). (C) Schematic representation of GST-fusion proteins of syntaxin 6 and VAMP3 used in GST pull-down assay. (D) Glutathione beads containing GST or GST-fusion proteins were incubated with His-MAR2_C. Resin-bound proteins (one-third of the eluate) were separated by SDS-PAGE and visualized by silver staining for GST or GST-fusion proteins (top) or by immunoblotting with anti-MAR2_C#41 for His-MAR2_C (bottom). One-tenth of the amount of His-MAR2_C used in the binding reaction was run in parallel (lane 1).

brane via the early/recycling endosomes (Ghosh *et al.*, 1998; Mallard *et al.*, 2002). In COS7 cells transfected with TGN38 alone, TGN38 was found to accumulate in the perinuclear region (Figure 6A, a). In contrast, most cells coexpressed with GFP-MAR2 showed the relocalization of TGN38 to the GFP-MAR2 vesicles (Figure 6A, b and b'). Next, antibody uptake assay was performed to monitor the cell surface-to-TGN transport of TGN38. The transfected living cells were incubated with an antibody recognizing the extracellular region of TGN38 (anti-TGN38) at 37°C for 40 min, during which time the anti-TGN38 bound to TGN38 arriving at the cell surface was internalized. After a 1-h chase at 37°C in basal medium, the fate of antibody/TGN38 complexes was determined. Whereas internalized antibodies seemed to reach the TGN in cells expressing TGN38 alone (Figure 6B, c), in the cotransfected cells they were accumulated in the GFP-MAR2 vesicles (Figure 6B, e and e'). A similar effect on TGN38 trafficking was observed in ~35% of the cells expressing the cytosolic domain of syntaxin 6 (Myc-syn6cyto), which is thought to act as a dominant negative mutant (Figure 6B, d) (Mallard *et al.*, 2002; Perera *et al.*, 2003; Kuliawat *et al.*, 2004). These results suggest that MARCH-II is involved in the early endosome-to-TGN transport pathway.

A further test was carried out with furin, a TGN-localized endoprotease cycling between the TGN and plasma membrane via the late endosome (Mallet and Maxfield, 1999).

COS7 cells were transfected with a furin construct in which the extracellular domain was replaced by a 3 × FLAG tag (FLAG-furin). At steady state, this construct was detected in the perinuclear region (Figure 6C, f) and the endocytosed anti-FLAG antibody was able to reach there (Figure 6D, h), as has been similarly reported with a tac-furin chimera (Ghosh *et al.*, 1998). In ~30% of the cells coexpressing GFP-MAR2 and either FLAG-furin or wild-type furin, an extensive colocalization was observed (Figures 6C, g and g', and S3) along with an accumulation of internalized anti-FLAG in the GFP-MAR2 vesicles (Figure 6D, j and j'). Interestingly, ~32% of cells expressing Myc-syn6cyto blocked anti-FLAG transport to the TGN (Figure 6D, i), suggesting that syntaxin 6 is involved in the late endosome-to-TGN transport pathway. The mannose 6-phosphate receptor also is known to take the late endosome-to-TGN transport pathway (Ghosh *et al.*, 2003). However, the staining pattern of 3 × FLAG-tagged mannose 6-phosphate receptor (MPR-FLAG) was only marginally affected by expression of GFP-MAR2 or Myc-syn6cyto (Figure S3). Nevertheless, some degree of overlap between MPR-FLAG and GFP-MAR2 could be observed in peripheral vesicles, probably the late endosomes (Figures 6E and S3). A triple-labeling study revealed that the relocalized furin was present in vesicles positive for both MPR-FLAG and GFP-MAR2 (Figure 6E) but not EEA1 (Figure 6F), suggesting that furin accumulated preferentially in the late en-

Figure 4. Recruitment of syntaxin 6 to the MARCH-II vesicles. (A–C) COS7 cells transfected with MARCH-II were double-stained with anti-MAR2_c#41 (1:1000) and a mouse monoclonal antibody against syntaxin 6 (A), EEA1 (B), or γ -adaptin (C) followed by Alexa 546-conjugated anti-rabbit IgG and Alexa 488-conjugated anti-mouse IgG. (D) COS7 cells transfected with GFP-MAR2 were stained with anti- β 1,4-galactosyltransferase followed by Alexa 546-conjugated anti-goat IgG. The signals were observed by confocal microscopy and those for MARCH-II/GFP-MAR2 and the marker proteins are shown as green and red, respectively. In contrast to the perinuclear localization in untransfected cells (triangles in A, merge), redistribution of syntaxin 6 to the MARCH-II endosomes is observed in cells overexpressing MARCH-II (asterisk). Asterisks and triangles, the position of nucleus. Bars, 10 μ m.

dosomal compartment under these conditions. Together, these data suggest that MARCH-II participates in the regulation of the retrograde transport to the TGN mediated by syntaxin 6.

MARCH-II Overexpression Perturbs the Localization and Transport of TfR

It was found that the majority of intracellular TfR also was relocalized to the MARCH-II vesicles by MARCH-II overexpression (Figure 7A), whereas Rab11 did not seem to have such redistribution (Figure 7B). MARCH-II may influence intracellular transport of TfR without affecting the structure of the perinuclear recycling endosome. To address this, we investigated the transport of Tf, which is taken up by the target cells through receptor-mediated endocytosis and returned to the cell surface via early/recycling endosomes (Gruenberg and Maxfield, 1995; Mellman, 1996). COS7 cells were transfected with FLAG-Rab11 (Figure 7C), GFP-MAR2 plus FLAG-Rab11 (Figure 7D), or MARCH-II (Figure 7E). Cells were labeled with TMR-labeled Tf (TMR-Tf) for 1 h. Internalized TMR-Tf was found to accumulate in the Rab11

compartment in cells not overexpressing MARCH-II (Figure 7C). In contrast, significant amounts of TMR-Tf and TfR were found in peripheral vesicles positive for MARCH-II/GFP-MAR2 (Figure 7, D and E), suggesting that MARCH-II overexpression blocks TfR transport to the recycling endosome.

Another observation was of a marked inhibition of Tf uptake (Figure 7F). The amounts of internalized Tf were measured by labeling cells with 125 I-Tf for 5, 15, 30, and 60 min. Cells transfected with MARCH-II showed an $\sim$ 20% reduction of uptake compared with control cells at all time points (Figure 7H). (Because the transfection efficiency was $\sim$ 50%, the real value for the reduction in the transfectants was estimated to be $\sim$ 40% of control.) The number and distribution of clathrin-coated vesicles, visualized by immunostaining with α -adaptin, were not substantially affected by expression of GFP-MAR2 (our unpublished data), eliminating the possibility that the reduced uptake of Tf was due to the inhibition of endocytosis per se by MARCH-II. Furthermore, a reduced surface expression of TfR was revealed by confocal immunofluorescence microscopy (Figure 7G)

Figure 5. Redistribution of syntaxin-6 partners. Right two panels, COS7 cells transfected with MARCH-II (A), FLAG-VAMP3 plus GFP-MAR2 (B), FLAG-VAMP4 plus GFP-MAR2 (C), or GFP-MAR2 (D). Left, control cells transfected with mock (A and D), FLAG-VAMP3 (B), or FLAG-VAMP4 (C). After 18 h of transfection, cells were processed for immunofluorescence analysis with antibodies for MARCH-II, Vti1a, FLAG-tag, and syntaxin 8 followed by Alexa 488-conjugated anti-rabbit IgG or TRITC-conjugated anti-mouse IgG. The signals for SNAREs are shown in red and those for MARCH-II/GFP-MAR2 are shown in green. Arrowheads indicate the colocalization of two proteins. Asterisks, the position of nucleus. Bars, 10 μ m.

and cell-surface biotinylation (Figure 7I, bottom). To measure the amounts of surface receptor, HeLa cells transfected with MARCH-II or an empty vector were labeled with 125 I-Tf on ice for 2 h. MARCH-II overexpression reduced the surface expression levels to $79 \pm 13\%$ of those in control cells ($p < 0.005$, $n = 9$), which is consistent with the reduction observed in Tf uptake. A similar observation has been reported with fluorescence-activated cell sorting (Bartee *et al.*, 2004). Therefore, inhibition of Tf uptake is caused by reduction of the surface expression levels of TfR. Unexpectedly, there was no change in the rates for internalization (K_e) and recycling (K_r) of Tf between pcDNA3- and MARCH-II-transfected HeLa cells (where $K_e = 0.206 \pm 0.014$ and $0.208 \pm 0.008 \text{ min}^{-1}$, respectively [$n = 6$ from two independent experiments]; $K_r = 0.046 \pm 0.004$ and $0.046 \pm 0.006 \text{ min}^{-1}$, respectively [$n = 4$ from two independent experiments]). Moreover, the total protein level of TfR was not affected (Figure 7I, top). Therefore, the reduction in surface TfR was not due to an alteration of the kinetics of TfR cycling or increased degradation of TfR. Taking account of the fact that most intracellular TfR was colocalized with overex-

pressed MARCH-II (Figure 7, A, E, and G), a certain population of TfR was likely trapped in the MARCH-II-positive compartments, leading to their absence on the cell surface. Together, the data suggest a contribution by MARCH-II to the endocytic recycling pathway.

Lack of Effect on the EGF/EGFR Down-Regulation and the Secretory Pathway

To examine the possible effects of MARCH-II on the endocytic transport to lysosomes, the endocytic transport of TMR-labeled EGF (TMR-epidermal growth factor) was monitored. Expression of GFP-MAR2 did not affect internalization of EGF compared with untransfected COS7 cells (our unpublished data). Endocytosed TMR-epidermal growth factor was initially present in peripheral small vesicles, corresponding to the early endosome (Figure 8, A and B) and was later concentrated in the GFP-MAR2 vesicles (Figure 8C, arrows). When cells were chased for 2 h at 37°C after a 30-min loading, TMR-epidermal growth factor was separated into distinct structures, probably lysosomes (Figure 8D). The degradation efficiency of EGFR was examined next. HeLa cells transfected with MARCH-II or an empty vector were stimulated with EGF for different time periods at 37°C and extracted. The levels of EGFR in each lysate were determined by immunoblot analysis. A similar rate of ligand-induced EGFR degradation was observed in both cells (Figure 8E). These results allow us to conclude that MARCH-II expression does not affect the degradative transport pathway to lysosomes.

The biosynthetic transport pathway was then examined using SEAP. COS7 cells were transfected with a SEAP expression vector together with either MARCH-II or an empty vector. Eighteen hours after transfection, culture medium was changed to a fresh one followed by incubation for 24 h at 37°C . The activity of SEAP in the medium was assayed. No difference in the activity was apparent between cells expressing SEAP alone ($304,100 \pm 45,200$ arbitrary fluorescence units of the reaction product, $n = 4$) and those expressing both SEAP and MARCH-II ($270,300 \pm 10,300$, $n = 6$). The activity value for mock-transfected cells was 42 ± 5 ($n = 2$).

Effects of Suppression of MARCH-II Expression

To further define the importance of MARCH-II on the endosomal transport, we determined the effects of depleting this protein by small interfering RNA (siRNA). HeLa cells stably expressing TGN38 (HeLa-TGN38) were further stably transfected with an shRNA expression vector targeted against MARCH-II. Single stable clones established by antibiotic selection were found to significantly reduce the expression levels of endogenous MARCH-II mRNA and overexpressed MARCH-II protein, reaching an average inhibition of 60% ($n = 15$) and 73% ($n = 24$) with respect to HeLa-TGN38 cells, respectively (Figure 9, A and B). MARCH-II shRNA was not associated with a change in the protein level of syntaxin 6, TGN38, TfR, or α -tubulin (Figure 9B). To determine whether depletion of MARCH-II would affect the retrograde TGN transport pathway, an antibody uptake assay was performed using anti-TGN38. In 12 of 24 single clones, $\sim 30\%$ of cells showed that endocytosed anti-TGN38 was accumulated in peripheral punctate structures, some of which were colocalized with the early/recycling endosomes labeled by 60-min uptake of Alexa 488-Tf, whereas their perinuclear accumulation was observed in control cells (Figure 9C). Of these clones, similar punctate staining of endogenous TGN46 at steady state, as determined by immunofluorescence microscopy, was observed in

Figure 6. Overexpression of MARCH-II perturbs the localization and transport of TGN38 and furin. (A) COS7 cells expressing TGN38 alone (a) or together with GFP-MAR2 (b and b') were fixed, permeabilized, and stained with anti-TGN38 followed by TRITC-conjugated secondary antibody. (B) Cells expressing TGN38 alone (c) or together with Myc-syn6cyto (d) or with GFP-MAR2 (e and e') were incubated with anti-TGN38 for 40 min and subsequently chased for 1 h at 37°C. Endocytosed antibodies were visualized with TRITC-conjugated secondary antibody. (C) Cells expressing FLAG-furin alone (f) or together with GFP-MAR2 (g and g') were fixed, permeabilized, and stained with anti-FLAG followed by TRITC-conjugated secondary antibody. (D) Cells expressing FLAG-furin alone (h) or together with Myc-syn6cyto (i) or with GFP-MAR2 (j and j') were incubated with anti-FLAG for 40 min and subsequently chased for 1 h at 37°C. Endocytosed antibodies were visualized with TRITC-conjugated secondary antibody. Arrowheads in (A–D) indicate the colocalization of two proteins. (E) Cells coexpressing furin, MPR-FLAG, and GFP-MAR2 were stained with anti-furin and anti-FLAG followed by Alexa 350- and TRITC-conjugated secondary antibodies, respectively. (F) Cells coexpressing furin and GFP-MAR2 were stained with anti-furin and anti-EEA1 followed by Alexa 350- and TRITC-conjugated secondary antibodies, respectively. Arrowheads in E and F indicate the positions of the vesicles positive for both GFP-MAR2 and furin. Asterisks, the position of nucleus. Bars, 10 μ m (A–D) or 5 μ m (E and F).

eight clones (in $\sim 30\%$ of cells) (Figure 9D). In contrast, no inhibition of TGN transport of anti-FLAG was detected in cells transfected with FLAG-furin (Figure 9E). Furthermore, uptake and transport of Alexa 488-Tf and TMR-epidermal growth factor seemed similar in HeLa-TGN38 cells and the shRNA-expressing clones (Figure 9, F and G), suggesting that the defects observed by MARCH-II shRNA were specific to the endosome-to-TGN pathway. To evaluate the possibility that the interference was caused by perturbation of syntaxin 6, we determined the cellular localization of syntaxin 6 in the clones that exhibit inhibition of anti-TGN38 transport. Strikingly, some redistribution of syntaxin 6 to the plasma membrane was observed in two clones; nevertheless the perinuclear TGN localization was still detected (Figure 9H). This was unlikely to be caused by disturbance of the Golgi/TGN and endosomes, as the staining patterns of β 1,4-galactosyltransferase, EEA1, TfR, and lamp-1 were indistinguishable from those of HeLa-TGN38 cells (Figures 9I and S4). In sum, these results suggest that MARCH-II is partially

required for proper distribution of syntaxin 6 as well as for maintenance of the endosomal transport pathway.

PDZ-binding Motif Is Essential for the Subcellular Localization of MARCH-II

The last four amino-acid residues of MARCH-II match a consensus class I PDZ-binding motif (-E-S/T-X-V/I, where X represents any amino acid) (Songyang *et al.*, 1997). GST pull-down assay detected the specific interaction between His-MAR2_C and Veli, a PDZ protein that binds to a class I motif (Jo *et al.*, 1999), which was abolished by replacing the Thr located two residues from the C terminus with Lys (His-MAR2_C-244K) (Figure 10A). When a GFP-tagged Thro-to-Lys mutant of MARCH-II (GFP-MAR2-244K) was transfected in COS7 cells, it exhibited a diffuse, reticular, and perinuclear expression pattern that colocalized with the ER marker calreticulin (Figure 10B); nevertheless, the peripheral punctate localization could still be observed, but less frequently than with wild-type MARCH-II (Fig-

Figure 7. MARCH-II overexpression affects Tf uptake and distribution of TfR. (A and G) COS7 cells transfected with MARCH-II were double stained with anti-MAR2_C#41 and anti-TfR, and the signals were observed with confocal microscopy. Images in G were focused on the cell surface area. (B) COS7 cells transfected with GFP-MAR2 were stained with anti-Rab11. (C–E) COS7 cells transfected with either FLAG-Rab11 (C), FLAG-Rab11 plus GFP-MAR2 (D), or MARCH-II (E) were incubated with TMR-Tf at 37°C for 1 h. Cells were washed, fixed, and stained with anti-FLAG (C and D) or with anti-MAR2_C#41 and anti-TfR (E). (F) COS7 cells expressing GFP-MAR2 were incubated with TMR-Tf at 37°C for 5 min (top) or 30 min (bottom). Cells were washed, fixed, and observed by fluorescence microscopy. Asterisks indicate cells expressing GFP-MAR2. Bars, 10 μm (A–G). (H) HeLa cells transfected with an MARCH-II (open circles) or an empty vector (closed circles) were incubated with ^{125}I -Tf at 37°C for 5, 15, 30, and 60 min. After washing with acidic buffer to release the cell surface-associated ^{125}I -Tf, the amounts of the internalized radioactivity were determined. Data are represented as the mean from three independent experiments. (I) HeLa cells were transfected with GFP (lanes 1 and 2) or GFP-MAR2 (lane 3). At 18 h after transfection, cells were incubated in HBSS (lane 1) or HBSS containing 0.5 mg/ml EZ-Link Sulfo-*N*-hydroxysuccinimide-LC-Biotin (lanes 2 and 3) on ice for 30 min. After washing with ice-cold 0.1 M Tris-HCl, pH 7.5, cells were extracted in radioimmunoprecipitation assay lysis buffer. Lysate (300 μg of proteins) was incubated with streptavidin-agarose beads and the bound materials (biotinylated cell surface proteins) were eluted by boiling in Laemmli sample buffer. Whole cell lysates (10 μg of proteins; top) and biotinylated samples (bottom) were subjected to immunoblot analysis with anti-TfR. Asterisk indicates dimeric forms of TfR.

ure 10C, left). The distribution of syntaxin 6, TGN46, and FLAG-furin was less affected and also overlapped with GFP-MAR2-244K in the perinuclear region (Figure 10, C and D; our unpublished data). Under these conditions, the inhibitory effects on Tf uptake and TGN transport of endocytosed antibodies were much lower than those observed in cells overexpressing wild-type MARCH-II (Figure 10E; our unpublished data). Based on the above-mentioned results, it is proposed that the PDZ-binding

motif of MARCH-II is important for the subcellular targeting required for effective activity in the endosomal transport pathway.

DISCUSSION

We here report the identification and characterization of a novel syntaxin-6-binding protein, MARCH-II, that is appar-

Figure 8. EGF trafficking and EGFR down-regulation are not affected by MARCH-II overexpression. (A–D) COS7 cells expressing GFP-MAR2 were incubated with TMR-epidermal growth factor for 5 min (A), 10 min (B), or 30 min (C) at 37°C. After a 30-min loading, cells were washed and incubated in marker-free medium for 2 h at 37°C (D). Merged images of fluorescence signals for GFP (green) and TMR (red) are shown. Overlaps of both signals are indicated by arrows. Bars, 20 μm. (E) HeLa cells transfected with MARCH-II (right) or an empty vector (left) were starved by a 1-h incubation in Opti-MEM I at 37°C. Cells were subsequently treated with 1 μg/ml EGF for indicated time at 37°C. Each sample (8 μg of protein) was subjected to immunoblot analysis with anti-EGFR (top), anti-calnexin (middle), and anti-MAR2_{C41} (bottom).

ently a component of the machinery regulating the endosomal trafficking.

Several lines of evidence support MARCH-II localizing to a subpopulation of endosomes: 1) subcellular fractionation revealed that MARCH-II was mainly present in the membranes enriched with endosomal proteins; 2) double-labeled confocal microscopy of CHO cells showed that MARCH-II was detected in peripheral vesicles, some of which were positive for syntaxin 6, TfR, EEA1, and γ-adaptin; and 3) when MARCH-II was overexpressed, the cycling proteins such as TGN38, chimeric furin, and Tf-TfR complex were accumulated into the vesicles positive for MARCH-II, whereas endocytosed EGF passed through and did not accumulate. The endosomal targeting is mediated by the C-terminal PDZ-binding motif, because site-directed mutagenesis of this motif was found to cause mislocalization of MARCH-II in the ER and Golgi/TGN.

Specific interaction of MARCH-II with syntaxin 6 was demonstrated by immunoprecipitation and *in vitro* binding studies. The complex formation occurred directly between the C-terminal tail of MARCH-II and the H2 domain of syntaxin 6. Subcellular fractionation and immunofluorescence analysis demonstrated that MARCH-II and syntaxin 6 not only differed in overall distribution patterns but also that some colocalization was evident. Thus, the two proteins are not present in the same compartment at all times and their

interaction may occur in restricted domains, perhaps intermediates between the TGN and endosomes. A number of SNARE-binding proteins, including Sec1/Munc18 family (Toonen and Verhage, 2003), Munc13 (Martin, 2002), and synaptotagmins (Südhof, 2002), regulate SNARE assembly and membrane trafficking (Gerst, 2003). It is possible that MARCH-II acts as a regulator of syntaxin-6-mediated fusion by determining the timing of SNARE complex formation and by modulating the stability of the complex.

Because a dramatic redistribution of syntaxin 6 was seen by overexpression and suppression of MARCH-II, MARCH-II is taken to influence a mechanism for the localization and trafficking of syntaxin 6. It is generally accepted that syntaxin 6 undergoes constitutive cycling between the TGN, endosomes, and plasma membrane (Chao *et al.*, 1999; Wendler and Tooze, 2001). The localization of syntaxin 6 is thought to be mediated by two independent cytosolic regions (Watson and Pessin, 2000). One is a tyrosine-based motif between the H1 and H2 domains (YGRL; residues 140–143), which acts as an internalization signal. The other the H2 domain as a TGN localization domain. However, target molecules for these motifs have not yet been found. MARCH-II might alternatively function as an endosomal retention receptor and/or sorting machinery for segregation from the endocytic pathway. If so, the following possible explanations for redistribution of syntaxin 6 caused by MARCH-II overexpression may be reasonably put forward: 1) most syntaxin 6 molecules were captured by MARCH-II and were unable to exit from endosomes; and 2) the H2 domain was masked by MARCH-II, thereby preventing the binding of other molecules for TGN retention and sorting.

Syntaxin 6 has been shown to be crucial for the early/recycling endosomes-to-TGN pathway (Mallard *et al.*, 2002). In addition, evidence is provided here for involvement of syntaxin 6 in the late endosome-to-TGN pathway. The cytosolic domain of syntaxin 6 blocked the TGN transport of both TGN38 and FLAG-furin. This is in agreement with the notion that syntaxin 6 participates in multiple trafficking pathways by association with a varied set of SNAREs (Wendler and Tooze, 2001). Similar inhibitory effects were observed by overexpression of MARCH-II. TGN38 and FLAG-furin were redistributed into peripheral vesicles at steady state, indicating that they were trapped into and unable to exit from the branch point before segregation away from the endocytic pathway. Perturbation of TGN delivery and localization seems unlikely to be due to disruption of the TGN membranes because the perinuclear localization of γ-adaptin and β1,4-galactosyltransferase was largely unaffected. These data imply close correlation of functional activities in the endosomes-to-TGN protein transport between MARCH-II and syntaxin 6; the transport interference would be due to the excess MARCH-II activity that acted as a dominant negative inhibitor of syntaxin 6, such as the cytosolic domain of syntaxin 6. This conclusion was supported by the observations that suppression of MARCH-II expression by siRNA partially affected the distribution of syntaxin 6 and TGN38/46.

The decrease in surface TfR by MARCH-II was first reported by Bartee *et al.* (2004) and is confirmed here by immunofluorescence microscopy and cell surface biotinylation assay. Unexpectedly, the rates of turnover and degradation of TfR were unchanged. The majority of TfR and internalized Tf was accumulated in the MARCH-II vesicles but not in the Rab11-positive perinuclear compartment, suggesting interference with transit of Tf-TfR from the early endosome to the recycling endosome. It seems likely that ~40% of the total receptor population was accumulated into

Figure 9. Specific effects of MARCH-II suppression on syntaxin 6 and TGN38/46. (A) Northern blot of HeLa-TGN38 cells (control) or those stably expressing the MARCH-II shRNA (siRNA) (20 μ g of total RNA each) was hybridized with a specific probe for human MARCH-II (top). The blot was subsequently reprobed with BiP/GRP78 cDNA as a loading control (bottom). (B) Control or the MARCH-II shRNA expressing HeLa-TGN38 cells were transiently transfected with human MARCH-II and lysed in radioimmunoprecipitation assay buffer at 18 h after transfection. Whole cell lysates (10 μ g of each protein) were subjected to immunoblot analysis with indicated antibodies. (C) HeLa-TGN38 cells (right) and those ex-

pressing the MARCH-II shRNA (left) were incubated with anti-TGN38 for 40 min and subsequently chased in presence of Alexa 488-Tf for 1 h at 37°C. Endocytosed antibodies were visualized with Alexa 546-conjugated secondary antibody. Right and left, signal for anti-TGN38. Middle, magnified image of inset in left image, which is merged with signals for anti-TGN38 (red) and Alexa 488-Tf (green). Arrowheads indicate colocalization of both signals. (D) HeLa-TGN38 cells (right) and those expressing the MARCH-II shRNA (left) were incubated with Alexa 488-Tf for 1 h at 37°C and subsequently stained with anti-TGN46 followed by Alexa 546-conjugated secondary antibody. Middle, magnified image of inset in left, which is merged with signals for anti-TGN46 (red) and Alexa 488-Tf (green). Arrowheads indicate colocalization of both signals. (E) HeLa-TGN38 cells expressing the MARCH-II shRNA were transiently transfected with FLAG-furin. Cells were incubated with anti-FLAG for 40 min and subsequently chased for 1 h at 37°C. Endocytosed antibodies were visualized with Alexa 546-conjugated secondary antibody. (F and G) HeLa-TGN38 cells expressing the MARCH-II shRNA were labeled by 1-h incubation with Alexa 488-Tf (F) or TMR-epidermal growth factor (G) at 37°C, and subsequently washed and fixed. Nucleus (blue) was labeled with Hoechst 33342. (H and I) HeLa-TGN38 cells (right) and those expressing the MARCH-II shRNA (left) were stained with anti-syntaxin 6 (H) or anti- β 1,4-galactosyltransferase (I) followed by Alexa 546-conjugated secondary antibody. Bars, 5 μ m (C and D) or 10 μ m (E–I).

the MARCH-II vesicles, whereas the remainder was normally cycling, but the mechanism remains unclear. Recycling back to the plasma membrane occurs directly from the early endosome (rapid cycle) or indirectly via the recycling endosome (slow cycle) (Ghosh and Maxfield, 1995; Ren *et al.*, 1998). One possibility is that MARCH-II overexpression blocked a slow cycle and an intact rapid one was able to recover Tf-TfR transport. However, there is no evidence for the contribution of syntaxin 6 to Tf-TfR transport. Considering the fact that VAMP3 is required for the targeted delivery of the Tf-TfR-containing vesicles to the plasma membrane (McMahon *et al.*, 1993; Galli *et al.*, 1994), the interference might be caused, in part, by redistribution of VAMP3.

Database searches revealed the presence of the *MARCH* genes, each with a PHD finger and two or more predicted transmembrane spans, in various eukaryotes from yeast to mammals (at least nine members exist in humans [MARCH-I to -IX]; Bartee *et al.*, 2004), suggesting conservation of function through evolution. γ -2 herpesviruses and poxviruses possess the genes encoding proteins (K3 and K5) that share a conserved domain structure similar to MARCHs (Früh *et al.*, 2002; Coscoy and Ganem, 2003). The PHD fingers of MARCHs and K3/K5 are structurally related to RING finger and possess E3 ubiquitin ligase activity (Aravind *et al.*, 2003; Dodd *et al.*, 2004). Doa10p, a yeast homologue of MARCH-VI, ubiquitinates short-lived proteins and participates in ER-associated degradation (Swanson *et al.*, 2001). MARCH-IV, MARCH-VIII/c-MIR, and MARCH-IX possess an E3 activity targeting major histocompatibility complex class I and a B7-2 costimulatory molecule for lysosomal degradation, suggesting an involvement in the regulation of immune recognition system (Goto *et al.*, 2003; Bartee *et al.*, 2004). Similarly, the viral K3/K5 down-regulates the immune recognition proteins, thereby enabling the virus to evade the host immune response (Früh *et al.*, 2002; Coscoy and Ganem, 2003). MARCH-II also is thought to function as a ubiquitin ligase because its PHD finger interacts with several ubiquitin-conjugating enzymes and displays auto-E3 activity in vitro (our unpublished data; Bartee *et al.*, 2004). In this regard, there is an interesting report that Staring (syn-

pressing the MARCH-II shRNA (left) were incubated with anti-TGN38 for 40 min and subsequently chased in presence of Alexa 488-Tf for 1 h at 37°C. Endocytosed antibodies were visualized with Alexa 546-conjugated secondary antibody. Right and left, signal for anti-TGN38. Middle, magnified image of inset in left image, which is merged with signals for anti-TGN38 (red) and Alexa 488-Tf (green). Arrowheads indicate colocalization of both signals. (D) HeLa-TGN38 cells (right) and those expressing the MARCH-II shRNA (left) were incubated with Alexa 488-Tf for 1 h at 37°C and subsequently stained with anti-TGN46 followed by Alexa 546-conjugated secondary antibody. Middle, magnified image of inset in left, which is merged with signals for anti-TGN46 (red) and Alexa 488-Tf (green). Arrowheads indicate colocalization of both signals. (E) HeLa-TGN38 cells expressing the MARCH-II shRNA were transiently transfected with FLAG-furin. Cells were incubated with anti-FLAG for 40 min and subsequently chased for 1 h at 37°C. Endocytosed antibodies were visualized with Alexa 546-conjugated secondary antibody. (F and G) HeLa-TGN38 cells expressing the MARCH-II shRNA were labeled by 1-h incubation with Alexa 488-Tf (F) or TMR-epidermal growth factor (G) at 37°C, and subsequently washed and fixed. Nucleus (blue) was labeled with Hoechst 33342. (H and I) HeLa-TGN38 cells (right) and those expressing the MARCH-II shRNA (left) were stained with anti-syntaxin 6 (H) or anti- β 1,4-galactosyltransferase (I) followed by Alexa 546-conjugated secondary antibody. Bars, 5 μ m (C and D) or 10 μ m (E–I).

Figure 10. Characterization of the PDZ-binding motif of MARCH-II. (A) GST pull-down assay. Glutathione beads containing GST or GST-Veli were incubated with either His-MAR2_C or His-MAR2_C-244K. Resin-bound proteins (1/15 of the eluates) were separated by SDS-PAGE and visualized by silver staining. One-tenth of the amount of His-MAR2_C or His-MAR2_C-244K used in the binding reaction was run in parallel (input). (B–D) COS7 cells transfected with GFP-MAR2-244K were processed for immunofluorescence analysis with an antibody against calreticulin (B), syntaxin 6 (C), or TGN46 (D) followed by Alexa 546-conjugated secondary antibody. (E) COS7 cells transfected with GFP-MAR2-244K were labeled with TMR-Tf for 30 min at 37°C. Cells were subsequently washed, fixed, and observed by fluorescence microscopy. The signals for GFP are shown as green and those for the marker proteins are shown as red. Bars, 10 μ m.

taxin-1-binding RING finger protein) acts as an E3 ligase targeting syntaxin 1 for degradation (Chin *et al.*, 2002), which raises the possibility that MARCH-II might control the activity of syntaxin 6 by a similar mechanism. Further work on the functional role of the PHD finger and identification of the PDZ protein that binds to MARCH-II will facilitate a better understanding of the membrane trafficking system of eukaryotic cells.

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