HM1.24 is Internalized from Lipid Rafts by Clathrin-mediated Endocytosis through Interaction with α-Adaptin*

Naoko Masuyama†, Toshio Kuronita†, Rika Tanaka‡, Tomonori Muto†, Yuko Hirota†, Azusa Takigawa‡, Hideaki Fujita¢, Yoshinori Aso†, Jun Amano§, and Yoshitaka Tanaka†‡

From the *Division of Pharmaceutical Cell Biology, Graduate School of Pharmaceutical Sciences, Kyushu University, 3-1-1 Maidashi, Fukuoka 812-8582 and †Chugai Pharmaceutical Company, Limited, Fuji-Gotemba Research Laboratories, 1-135 Komakado, Gotemba-shi, Shizuoka 412-8513, Japan

HM1.24/Bst2/CD317 is a protein highly expressed in multiple myeloma cells and has unique topology with two membrane anchor domains, an NH₂-terminal transmembrane domain and a glycosylphosphatidylinositol attached to the COOH terminus. We show here that human HM1.24 is localized not only on the cell surface but also in the trans-Golgi network and/or recycling endosomes, where it resides in detergent-resistant microdomains, lipid rafts. In contrast to other glycosylphosphatidylinositol-anchored proteins, HM1.24 was internalized from lipid rafts on the cell surface by clathrin-mediated endocytosis. Interestingly, a non-canonical tyrosine-based motif, which contains two tyrosine residues, Tyr-6 and Tyr-8, present in the NH₂-terminal cytoplasmic domain of HM1.24, was essential for endocytosis through interaction with an α-adaptin, but not μ2-subunit, of the AP-2 complex. Indeed, an appendage domain of α-adaptin was identified as a protein interacting with the cytoplasmic tail of HM1.24. Furthermore, overexpression of the appendage domain of α-adaptin in cells depleted of α-adaptin could rescue the clathrin-mediated endocytosis of HM1.24 but not of the transferrin receptor. Taken together, our findings suggest that clathrin-dependent endocytosis of human HM1.24 from the cell surface lipid rafts is mediated by direct interaction with α-adaptin.

HM1.24 was originally identified as a plasma cell-specific antigen by a mouse monoclonal antibody generated for the development of a novel immunotherapeutic strategy for multiple myeloma cells (1). Based on amino acid sequences deduced from cDNA cloning (2), it was found that HM1.24 is identical to BST2, which was identified as a bone marrow stromal cell antigen (3). It has been proposed that HM1.24 serves as a target molecule for myeloma immunotherapy, because it is expressed preferentially on terminally differentiated normal and neoplastic B cells (4). Indeed, mouse or humanized monoclonal antibodies to HM1.24 induces potent antitumor cell activity by antibody-dependent cellular cytotoxicity in vivo or in vitro (1). Recently, HM1.24 was designated CD317 and found to be highly expressed in all B cell stages of differentiation, on bone marrow CD34⁺ cells, and also on T cells (6). In addition to myeloma cells, overexpression of HM1.24 has also been described in a wide variety of invasive solid tumor cell lines (7), in pancreatic ductal adenocarcinoma (8), and in pancreatic endocrine tumors (9). Although the mechanism by which HM1.24 is overexpressed remains to be elucidated, in the promoter region of the HM1.24 gene (BST2), a tandem repeat of three cis elements for a transcription factor, signal transducers and activators of transcription 3, which mediates interleukin-6 response gene expression, is contained upstream of the transcription initiation site. By the generation of monoclonal antibodies to mouse type I IFN⁴-producing cells, it was revealed that HM1.24 is predominantly expressed in IFN-producing cell in the native mouse, but is up-regulated on most cell types following stimulation with type I IFNs and IFNγ (10). In IFN-producing cells, monoclonal antibodies against HM1.24 abrogate IFNα secretion in response to Toll-like receptor stimuli CpG, suggesting that it may be involved in the secretion of cytokines. Recently, HM1.24 has been identified as the IFNo-induced restriction factor that inhibits HIV particle release from the cell surface, and is counteracted by the HIV-1 accessory protein, Vpu (11, 12).

Human HM1.24 has 180 amino acids and was originally postulated as a type II transmembrane protein, which is composed of a 21-amino acid NH₂-terminal cytoplasmic domain, followed by a 22-amino acid transmembrane domain and a large extracellular domain containing two possible N-glycosylation sites, giving a molecular mass of 29–33 kDa. By immunoscreening of a rat liver cDNA expression library, Kupzig et al. (13) have identified the rat homolog of human HM1.24. Rat HM1.24 is anchored in lipid rafts at the cell surface via a putative COOH-terminal glycosyl-phosphatidylinositol (GPI), but the transmembrane domain near the NH₂ terminus lies outside the lipid membrane.
α-Adaptin-dependent HM1.24 Endocytosis

rafts (13), thereby possessing unique topology. Rat HM1.24 localizes to, and continuously cycles between, the cell surface and the TGN. On the other hand, human HM1.24 shares only ~34 and 36% identity with rat and hamster homologues, respectively (13, 14). Hamster HM1.24, identified as a Golgi-resident GPI-anchored protein, has been reported to be involved in maintenance of the Golgi structure (14). Golgi localization of Golgi-resident GPI-anchored protein requires a tandem repeat of the EQ sequence, which is not contained in other homologs; therefore, divergence of the HM1.24 sequence among species may cause differences in its cellular function and/or biological property.

While this report was in preparation, Rollason et al. (15) reported that rat HM1.24 is internalized from the cell surface in a clathrin-dependent manner. Internalization of rat HM1.24 is dependent upon a dual tyrosine motif in its NH2-terminal cytoplasmic domain, which is recognized by the μ2-subunit of AP-2 complex. Moreover, after endocytosis rat HM1.24 delivered to an early endosome is subsequently transported to the TGN through recognition of the cytoplasmic domain by the μ1-subunit of AP-1 complex, suggesting the involvement of the sequential action of AP-2 and AP-1 complexes in internalization and delivery back to the TGN of rat HM1.24. Despite the critical requirement of the NH2-terminal cytoplasmic tail lying outside lipid rafts for trafficking of rat HM1.24, lipid raft association is required for efficient internalization of rat HM1.24.

Consistent with rat HM1.24 (15), we also show that human HM1.24 localizes to the cell surface and internal compartments, presumably the TGN and/or recycling endosomes, and is internalized from lipid rafts on the cell surface in a clathrin-dependent manner with a non-canonical dual Tyr motif. Notably, we have identified the appendage domain of α-adaptin as an interacting partner for the cytoplasmic tail of HM1.24. Furthermore, the appendage domain of α-adaptin specifically recognized the dual tyrosine motif and was necessary and sufficient for the clathrin-dependent endocytosis of HM1.24 from lipid rafts.

EXPERIMENTAL PROCEDURES

Materials—Culture media and fetal bovine serum (FBS) were purchased from GIBCO Invitrogen. Alexa488-conjugated human transferrin (Tfn), Texas Red-conjugated cholera toxin subunit B, tetramethylrhodamine (Rh)-conjugated epidermal growth factor (EGF), and Alexa488- and Alexa594-labeled secondary antibodies were purchased from Molecular Probes. EXPRESS™ Protein Labeling Mix, 35S-Easy Tag™, was obtained from New England Nuclear. Sulfo-NHS-SS-biotin and Streptavidin-Sepharose were from Pierce. Nystatin, methyl-β-cyclodextrin (MβCD), and Protein A-agarose were from Sigma-Aldrich.

Cell Culture—ARH77 and U937 cells were cultured in RPMI1640 supplemented with 10% FBS, 2 mM glutamine, and 1% penicillin-streptomycin. HeLa, normal human fibroblasts, A431, U937, and COS cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS, 2 mM glutamine, and 1% penicillin-streptomycin in humidified 95% air and 5% CO2 at 37 °C. For immunofluorescence experiments, the cells were plated onto 13-mm coverslips the day before transfection. After 36 h, the cells were used for immunocytochemical experiments.

Antibodies—Humanized monoclonal antibodies against HM1.24 carrying the Fc region derived from human IgG1κ were described previously (16). Rabbit polyclonal antibodies to HM1.24 were raised against glutathione S-transferase (GST) fusion protein of the extracellular domain corresponding to amino acid residues 49–162. Mouse monoclonal antibodies to human LAMP-1 and LAMP-2 were obtained from the Developmental Studies Hybridoma Bank maintained by the University of Iowa. Mouse monoclonal antibodies to γ-adaptin, α-adaptin, β2-adaptin, μ2-subunit (AP50), EEA1, GM130, flotillin, and rabbit polyclonal antibodies to caveolin-1 were purchased from BD Transduction Laboratories. Mouse monoclonal antibodies to CD59 and FLAG-M2 and -M5 were obtained from Calbiochem and Sigma, respectively. Mouse monoclonal antibodies to TfnR were purchased from Zymed Laboratories Inc.. Rabbit polyclonal antibodies to rat cation-independent mannose 6-phosphate/insulin-like growth factor-II receptor (MRP300) were described previously (17).

Immunofluorescence Microscopy—Immunofluorescence analysis was performed as described previously (18). Briefly, cells cultured on coverslips were fixed immediately in 4% paraformaldehyde in PBS, pH 7.4, for 30 min at room temperature, and permeabilized with 0.05% saponin in PBS for 15 min. Cells were quenched with 50 mM NH4Cl in PBS for 15 min and blocked with 1% bovine serum albumin in PBS for 30 min. The cells were then incubated for 1 h in the primary antibody diluted in blocking solution, as described previously (19). The cells were incubated for 30 min with Alexa488-, Cy3-, or Cy5-labeled secondary antibodies. Coverslips were then mounted in Mowiol onto glass slides, and the cells were analyzed by a confocal laser scanning microscope using a Zeiss LMS 510 META equipped with an argon/HeNe laser and a Zeiss 100×/1.4 Plan-Apochromat oil immersion lens. Photographic images were processed using Photoshop (Adobe Systems).

Western Blotting—Cells cultured on 35-mm dishes were washed with PBS, scraped, and homogenized in PBS/1% Triton X-100 containing protease inhibitor mixture. 100 μg of protein was subjected to SDS-PAGE (10% polyacrylamide) under reducing conditions. Proteins were transferred to a polyvinylidene difluoride membrane (Schleicher and Schüll), which was subsequently blocked with 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% Triton X-100 (TBS-T), 5% milk powder for 1 h at room temperature. The blot was incubated overnight at 4 °C with rabbit anti-human HM1.24, followed by washing with TBS-T. Subsequently, incubation with horseradish peroxidase-coupled goat anti-rabbit antibody was performed for 1 h at room temperature followed by washing with TBS-T. Blots were finally analyzed using the ECL Detection System (Amersham Biosciences).

Isolation of Lipid Rafts—HeLa, ARH77, and U937 cells were suspended in 1% Triton X-100 containing TKM buffer (50 mM Tris-HCl, pH 7.4, 25 mM KCl, 5 mM MgCl2, 1 mM EDTA, and protease inhibitor mixture) and incubated on ice for 30 min. The cell lysates were then mixed with an equal volume of 80% sucrose in TKM buffer. The mixture was transferred to a centrifuge tube and sequentially overlaid with 35 and 5% sucrose in
TKM buffer. The discontinuous sucrose gradient was centrifuged at 160,000 × g for 20 h in an SW65 rotor (Hitachi Co.), and 0.5-ml fractions were collected from the top of the tube.

**Antibody Uptake and Endocytosis Assays**—HeLa cells were incubated with the antibody to HM1.24 (2 µg/ml) for 15 min at 4 °C. For Tfna internalization, cells cultured on coverslips were incubated in serum-free Dulbecco’s modified Eagle’s medium with 1 mg/ml bovine serum albumin for 30 min and then with Alexa488- or Cy3-labeled Tfna (25 µg/ml) for 15 min at 4 °C. Cells were washed twice with PBS at 4 °C and further incubated in Dulbecco’s modified Eagle’s medium containing 10% FBS at 37 °C for the indicated time periods. After internalization, HM1.24 antibody and Alexa488- or Cy3-labeled Tfna present on the cell surface were removed by 3 × 10 min in 0.15 M NaCl, 0.1 M glycine-HCl buffer, pH 3, and cells were fixed in 4% paraformaldehyde in PBS.

**Treatment with Triton X-100 of Living Cells**—HeLa cells were incubated with the antibody to HM1.24 (2 µg/ml) and Alexa488-Tfna (50 µg/ml) for 15 min at 4 °C. Cells were washed twice with PBS at 4 °C and further incubated in Dulbecco’s modified Eagle’s medium containing 10% FBS at 37 °C for 5 min. HM1.24 antibody and Alexa488-Tfna present on the cell surface were removed by acid wash, and cells were left in ice-cold 1% Triton X-100 in PBS for 20 min at 4 °C, followed by fixation with 4% paraformaldehyde at room temperature.

**K⁺-depletion, Nystatin, and MβCD Treatment**—Pharmacological treatment was performed as described previously by Di Guglielmo et al. (20) except that nystatin and MβCD were used at 25 µM and 10 mM, respectively.

**Plasmids**—The complete open reading frame of human HM1.24 was PCR-amplified from the HeLa cell cDNA library using specific primers and was cloned into the BamHI/KpnI restriction sites of pcDNA3.1 vector (Invitrogen) by adding appropriate restriction sites to PCR primers. Point mutations of the HM1.24 tail were generated with the QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer’s manual. For the construct of HM1.24dNT, the NH₂-terminal cleavable signal sequence of rat LGP107 corresponding to amino acid residues 1–23 and the extracellular domain DIII corresponding to amino acid residues 529–897, the full-length Dyn2 was amplified by PCR from the human brain cDNA library and cloned into the BglII/KpnI of pEGFP-N1. Dyn2-K44A was generated with the QuikChange site-directed mutagenesis kit. The AP-2 α-adaptin appendage domain corresponding to amino acid residues 700–939 (α700–939) or 793–939 (α793–939) was amplified by PCR using full-length human AP-2 α-adaptin (Open Biosystem) as a template. FLAG-tagged α700–939 or α793–939 was cloned into p3XFLAG-CMV-7.1 vector (Sigma-Aldrich). The DNA sequence of the constructs was always confirmed by dyeoxy chain reaction termination sequencing, using the DNA Sequencing kit (BigDye™ Termination Cycle Sequencing Ready Reaction, ABI PRISM) and ABI PRISM 310 Genetic Analyzer (ABI PRISM). Transfections were carried out with FuGENE6 (Roche Molecular Biochemicals) according to the manufacturer’s instructions. After 36 h, the cells were used for immunocytochemical or biochemical experiments.

**Yeast Two-hybrid Assays**—The MATCHMAKER Two-Hybrid 3 (Clontech) was used. The cytoplasmic tail of HM1.24 corresponding to amino acid residues 1–21 was cloned into pGBK17, and the plasmid was transformed into the yeast strain AH109 following by screening a pACT2 HeLa cell cDNA library (Clontech). For interaction of the cytoplasmic tail HM1.24 with μ1A-subunit, μ2-subunit, or α-adaptin appendage domain, DNA fragments corresponding to the cytoplasmic tail of HM1.24 were cloned into pGBK17, cotransfected with pGADT7-μ1A-subunit, pGADT7-μ2-subunit, or pGADT7-α-adaptin appendage domain into yeast strain AH109. Constructs encoding full-length μ1A- and μ2-subunits (a kind gift from Dr. J. S. Bonifacino, National Institutes of Health) or fragments of α-adaptin were generated by PCR using plasmid DNA as a template. Transformation of yeast AH109 cells with the indicated constructs was performed by the lithium acetate procedure as described in the instructions for the MATCHMAKER two-hybrid kit (Clontech). The transformed yeast cells were isolated by growth on defined medium lacking leucine and tryptophan. Protein-protein interaction was monitored by growing on medium lacking leucine and tryptophan, leucine, tryptophan, and histidine, or leucine, tryptophan, histidine, and adenine at 30 °C for 3–4 days.

**RNA-mediated Interference**—The following small-interfering RNA (siRNA) oligonucleotides synthesized by B-Bridge International were used: AUCCGCGCGAUAGUACGUATT for negative control, UCCAAUUCGAAGACCAAUUTT for clathrin heavy chain (CHC), GUGGAUGCCUUUCGGGU- CATT for μ2-subunit, GAGCAUGUGCACGUGGCCATT for α-adaptin, GGCAUCAAGUACGGAAGATT for μ1A-subunit (22), AAAGUGCUUGAAGAUAGGAGUATT for β2-adaptin (23), and AGCCGAGCUGAGGAGAGCA for caveolin-1 (24). HeLa cells were transfected with the indicated siRNAs using Oligofectamine (Invitrogen) according to the manufacturer’s protocol. Experiments were performed 48–72 h after siRNA transection.

**RESULTS**

**Localization of Human HM1.24**—We first examined the expression and localization of HM1.24 in several human cell lines using a humanized monoclonal antibody specific for human HM1.24 (1). As shown in Fig. 1, the expression of HM1.24 was found in human myeloma cells, ARH77 and U937, HeLa, and A431 cells, but not in HEK293, U251, and human skin fibroblast cell lines, in both immunofluorescence and Western blot analyses, indicating the cell-type specific expression of HM1.24. As previously described (2), two broad bands with molecular masses ranging from 29 to 33 and 50 to 66 kDa, which correspond to the monomer and dimer, respectively,
were detected. In these cells, HM1.24 was distributed both on the cell surface and around the perinuclear region in immunofluorescence analyses, consistent with the results reported previously (13).

To examine the perinuclear localization of HM1.24, we carried out double staining experiments using several organelle-specific antibodies. To this end, we used HeLa cells, which have a larger cytoplasm than the other cell lines used here, making it easier to distinguish the perinuclear localization of HM1.24. The perinuclear localization of HM1.24 partially co-localized with a cis-Golgi marker GM130, the TGN marker GGA3, and a recycling endosome marker TfnR (Fig. 2). Little HM1.24 was, however, seen in vesicular compartments positive for a late endosome/lysosome marker LAMP-2 or an early endosome marker EEA1, indicating that HM1.24 is localized to the TGN and/or recycling endosomes, as well as the cell surface.

**HM1.24 Is Localized in Lipid Rafts/Detergent-insoluble Membrane Microdomains**—Such a dual localization of HM1.24 also partially overlapped with caveolin-1 (Fig. 2), which is localized to the cell surface and TGN and/or endosomes as well as caveosomes (25). Caveolin-1 associates with lipid rafts that have been referred to as a specific membrane microdomain enriched with cholesterol and glycosphingolipids (26). Proteins associated with lipid rafts have the characteristic of resistance to solubilization by nonionic detergent, such as Triton X-100 (27). In fact, <10% of total HM1.24 was solubilized by 1% Triton X-100. Effective solubilization of HM1.24 was achieved with 1% SDS (Fig. 3A). To further assess whether HM1.24 resides in lipid rafts, HeLa, ARH77, or U937 cells were lysed with 1% Triton X-100 at 4 °C and subjected to discontinuous sucrose density centrifugation. As shown in Fig. 3B, HM1.24 was predominantly detected in low density fractions, where both caveolin-1 and flotillin-1, lipid raft markers (28, 29), were also recovered. Because the expression of caveolin-1 was very low in ARH77 cells and undetectable in U937 cells (Fig. 1C), flotillin-1 was used as a lipid raft marker in these cells. Regardless of the result that ~30% of total HM1.24 was expressed on the cell surface when assayed by the biotinylation of cell surface proteins (data not shown), >95% of HM1.24 was recovered from lipid raft fractions. It is suggested, therefore, that HM1.24 localized to not only the cell surface but also to the TGN resides in lipid rafts. The results of Fig. 3C further show that the GPI-anchor domain of HM1.24 is necessary for its localization to lipid rafts. Glycosylphosphatidylinositol-specific phospholipase C (PI-PLC) treatment of cells increased the

**FIGURE 1.** Expression and localization of HM1.24 in various human cell lines. A, schematic representation of human and rat HM1.24 structure. Numbers correspond to amino acids. B, ARH77, U937, HeLa, A431, HEK239, U251, and human skin fibroblast cells cultured on coverslips were fixed, permeabilized, and incubated with HM1.24 antibodies. Subsequently, cells were incubated with Alexa488-labeled goat anti-mouse IgG and visualized by confocal microscopy. Bar, 20 μm. C, these cells cultured on 35-mm dish were lysed, and HM1.24 was immunoprecipitated with anti-HM1.24 antibody. The immunoprecipitates were the analyzed by immunoblotting with anti-HM1.24 antibody. Each 100 μg of protein was directly subjected to SDS-PAGE, followed by immunoblotting for caveolin-1 or flotillin-1.

**FIGURE 2.** Intracellular localization of HM1.24. HeLa cells were fixed, permeabilized, and incubated with primary antibodies to HM1.24 and EEA1, LAMP-1, Caveolin-1, GM130, GGA3, or TfnR. The primary antibodies were revealed by incubation with either Alexa488-conjugated anti-rabbit immunoglobulin or Cy3-conjugated anti-mouse immunoglobulin secondary antibodies. Cells were visualized by confocal microscopy. Right columns show the merged images for double staining of HM1.24 (green) and each organelle marker (red). Squares indicate magnified regions (mag.). Bars, 10 μm.
FIGURE 3. Lipid raft localization of HM1.24. A, HeLa cells were lysed with 1% Triton X-100 or 1% SDS on ice for 30 min, and each detergent-solubilized fraction was subjected to SDS-PAGE and Western blotting for HM1.24, LAMP-1, and caveolin-1, as described in Fig. 2. B, HeLa, ARH77, and U973 cells were lysed with 1% Triton X-100 containing buffer on ice for 30 min and subjected to discontinuous sucrose density gradient as described under "Experimental Procedures." After ultracentrifugation, each fraction was directly resolved on SDS-PAGE and analyzed by Western blot to detect HM1.24, LAMP-1, and caveolin-1 (HeLa cell) or flotillin-1 (ARH77 and U973 cells). Low density fractions (2–4) contained detergent-insoluble lipid raft fractions. C, HeLa cells were incubated in media with or without PI-PLC (0.5 unit/ml) at 37 °C for 30 min and lysed with 1% Triton X-100 on ice for 30 min. Cells were then centrifuged, and the resulting soluble (sup) and insoluble (ppt) fractions were analyzed by SDS-PAGE and immunoblotting for HM1.24, TfnR, and caveolin-1, as described in Fig. 1. IgG-HC, IgG heavy chain.

amount of HM1.24 in Triton X-100 extracts, whereas a non-GPI protein, caveolin-1, was resistant to Triton X-100, even after PI-PLC treatment.

Internalization of Anti-HM1.24 Antibody—Despite the lipid raft localization, the distribution of HM1.24 partially overlapped TfnR. To address whether this reflects the endocytic pathway of HM1.24, cells preincubated with HM1.24 antibody and Cy3-Tfn or Rh-EGF at 4 °C were further chased for 10 or 30 min at 37 °C, and distribution of the internalized antibody was compared with that of internalized Cy3-Tfn or Rh-EGF using confocal laser microscopy. To visualize the antibody internalized at 37 °C, the surface-bound HM1.24 antibody was stripped by an acid wash, which was sufficient to remove >90% of surface-bound HM1.24 antibody (data not shown). Despite some punctuate staining of HM1.24 antibody on the cell surface co-localized with Cy3-Tfn and Rh-EGF, after 10 and 30 min of internalization, most of the HM1.24 antibody was seen in numerous small vesicles around the perinuclear region, where it co-localized extensively with Cy3-Tfn internalized simultaneously (Fig. 4A). Such co-localization between the HM1.24 antibody and Alexa488-Tfn has already been seen in numerous small vesicles at 5 min after internalization (see Fig. 4B). On the other hand, some internalized HM1.24 antibodies co-localized with Rh-EGF after 10 min of internalization, whereas they were delivered to distinct vesicular compartments after 30 min of internalization (Fig. 4A), suggesting that, after internalization, HM1.24 is mainly transported to recycling endosomes and/or the TGN without being significantly delivered to late endosomes/lysosomes, to which endocytosed EGF is destined to be degraded.

To exclude the possibility that the internalization of HM1.24 was merely triggered by binding with antibodies, we performed a cell surface biotinylation assay. HeLa cells, which were labeled with [35S]methionine/cysteine for 1 h and chased for 1 h, the time necessary for the maximum appearance of newly synthesized HM1.24 on the cell surface (supplemental Fig. S1, A and B), were biotinylated with cleavable, membrane-impermeable sulfo-NHS-SS biotin and further incubated at 37 °C for varying times to allow internalization. After chasing for the indicated times, biotins remaining on the cell surface were cleaved by membrane-impermeable sodium 2-mercaptoethanesulfonate to detect only internalized molecules.

Because the precursor of HM1.24, which is an endoglycosidase H-sensitive endoplasmic reticulum form, was not biotinylated (supplemental Fig. S1A), it is suggested that only extracellular portion of HM1.24 was labeled with biotin. HM1.24 biotinylated at the cell surface acquired sodium 2-mercaptoethanesulfonate resistance depending on the chase time (supplemental Fig. S1, C and D). After 3 h of chasing, ~60% of the cell surface-expressing HM1.24 acquired resistance to sodium 2-mercaptoethanesulfonate, thereby suggesting that HM1.24 is constitutively endocytosed from the cell surface.

We further examined whether rapid co-localization between internalized HM1.24 antibody and Alexa488-Tfn reflects the lateral mobility of HM1.24 from lipid rafts to non-lipid rafts prior to endocytosis. Cells preincubated with HM1.24 antibody and Alexa488-Tfn at 4 °C were chased for 0 and 5 min at 37 °C and were subsequently treated with 1% Triton X-100 at 4 °C prior to fixation. As expected, HM1.24 antibody bound to the cell surface was significantly resistant to Triton X-100 treatment, but Alexa488-Tfn almost disappeared (Fig. 4B), confirming the localization of HM1.24 in lipid rafts on the cell surface.
**α-Adaptin-dependent HM1.24 Endocytosis**

![Diagram](image)

**FIGURE 4. Internalization of HM1.24 from lipid rafts.** A, HeLa cells were incubated with anti-HM1.24 antibody (green) and Cy3-Tfn or Rh-EGF (red) 4 °C for 30 min, fixed, and labeled HM1.24 antibody with Alexa488-secondary antibody (bound). For internalization experiments, HeLa cells preincubated with HM1.24 antibody (green) and Cy3-Tfn or Rh-EGF (red) for 30 min at 4 °C were further chased at 37 °C for 10 or 30 min. Non-internalized ligand was removed by washing at pH 3.0, 4 °C. Then, cells were fixed, permeabilized to detect the internalized antibody, and stained with Alexa488-conjugated secondary antibody (bound). For internalization experiments, HeLa cells preincubated with Alexa488-Tfn (4 °C) at 4 °C for 30 min were or were not chased for 5 min at 37 °C and then incubated at 4 °C for 10 min with PBS alone or containing 1% Triton X-100 prior to fixation. Non-internalized ligand was removed by washing at pH 3.0, 4 °C before treatment with Triton X-100. Cells were fixed, incubated with Cy3-conjugated secondary antibody, and visualized by confocal microscopy. Bars, 10 μm.

After 5-min internalization, most of the HM1.24 antibody had already co-localized with Alexa488-Tfn. Although treatment of living cells with Triton X-100 caused complete disappearance of the internalized Alexa488-Tfn, the internalized HM1.24 antibody was not significantly affected, indicating that HM1.24 is present in a detergent-resistant microdomain of early endosomes containing internalized Tfn. These results suggest that HM1.24 associated with lipid rafts is internalized through structures that maintain detergent resistance in the early steps of endocytosis.

**Clathrin-dependent Endocytosis of HM1.24**—The internalization of GPI anchor proteins has been thought to be mediated in a dynamin (Dyn)-independent manner (30–32); therefore, a dominant-negative mutant of Dyn, DynK44A, blocks both clathrin-dependent and -independent, but not caveolaoid/lipid raft-dependent, endocytosis (33). We then examined the effect of Dyn2K44A on the endocytosis of HM1.24 from the cell surface in comparison with that of TfnR or EGFR and a GPI-anchor protein CD59, which are endocytosed in a Dyn-dependent and -independent manner (32, 34), respectively. As shown in Fig. 5A, although transient expression of the GFP-Dyn2 wild type in HeLa cells had no effect on the internalization of all probes used, GFP-Dyn2K44A significantly inhibited the internalization of Cy3-Tfn, Rh-EGF, and HM1.24 antibody, but not CD59 antibody.

We further investigated the internalization mechanism of HM1.24 by using pharmacological inhibitors, such as potassium depletion, for clathrin-dependent endocytosis (35) and MβCD or nystatin for caveolar/lipid-raft-dependent endocytosis (36). Uptake of Alexa488-Tfn was severely inhibited by potassium depletion (supplemental Fig. S2A), but not by MβCD and nystatin, although MβCD treatment also caused partial reduction of endocytosis of Cy3-Tfn (Fig. 5B) and Rh-EGF (supplemental Fig. S2B), in accordance with the fact that clathrin-dependent endocytosis is also affected by MβCD (37, 38). Similarly, HM1.24 antibody uptake was almost completely inhibited by potassium depletion (supplemental Fig. S2A), which nevertheless had no inhibitory effect on the internalization of CD59 antibody (data not shown). Furthermore, neither nystatin nor MβCD significantly influenced the internalization of HM1.24 antibody, whereas MβCD partially inhibited HM1.24 antibody uptake, as seen in Cy3-Tfn. Nevertheless, both drugs blocked the uptake of the cell surface-bound CD59 antibody (Fig. 5C) and Texas Red-CTxB (supplemental Fig. S2C), both of which are present in lipid rafts and endocytosed by a clathrin-independent mechanism (39). These results suggest, therefore, that the mechanism by which HM1.24 is internalized depends on clathrin. Involvement of clathrin in the endocytosis of HM1.24 was further demonstrated in cells depleted of CHC expression by siRNA. The expression levels of CHC in HeLa cells transfected with siRNA specific to CHC were reduced to <10% of those in control cells, as analyzed by Western blotting (Fig. 6A). Consistent with previous findings (40–42), the internalization of both Alexa488-Tfn and Rh-EGF was significantly blocked in CHC-depleted cells (Fig. 6, D and E). As expected, the endocytosis of HM1.24 antibody was also strongly inhibited in cells depleted of CHC (Fig. 6, B and C). In addition, depletion of caveolin-1 by siRNA did not block HM1.24 antibody uptake (Fig. 6B). Taken together, these results apparently indicate that HM1.24 is endocytosed in a clathrin-dependent manner.

**Two Tyr Residues Present in the NH2-terminal Cytoplasmic Tail Are Necessary for the Internalization of HM1.24 from the Cell Surface**—Proteins endocytosed by the clathrin-dependent process contain the information necessary for internalization in their cytoplasmic domain (43). So far, three signals are known to be involved in clathrin-dependent endocytosis; a Y-based motif (YXXΦ), an NPXY motif, and a dileucine-based motif ([DE][XX][L/I]). Indeed, the NH2-terminal cytoplasmic tail of HM1.24, consisting of 21 amino acids, contains two Tyr residues, at positions 6 and 8 from the NH2 terminus, and the latter constitutes a typical Y-based type motif, YCRV. We first examined whether the cytoplasmic tail, but not COOH-terminal GPI anchor, is the predominant domain that determines the local-
examined the internalization of HM1.24 antibody in cells expressing these deletion mutants. Although the antibody was found to be efficiently delivered to the perinuclear region in cells expressing either the wild type or dCT, it stayed exclusively retained on the cell surface in cells expressing dNT even after 30-min internalization periods (Fig. 7B). These results suggest that the NH2-terminal cytoplasmic and/or following transmembrane domains are determinants in the localization to, and trafficking between, the cell surface and perinuclear region.

To further assess the involvement of either or both Tyr residues in the endocytosis of HM1.24, two Tyr residues were substituted individually, or in combination, to the alanine residue, and were transiently expressed in COS cells. As shown in Fig. 7B, mutation of each single Tyr residue, Y6A and Y8A, did not significantly affect not only the steady-state distribution, but also internalization from the cell surface, of HM1.24, although the expression of both mutants on the cell surface showed a tendency to slightly increase as compared with WT. Interestingly, the double mutation of both Tyr residues (Y6A,Y8A) considerably abrogated the perinuclear localization of HM1.24. Consequently, Y6A,Y8A was distributed exclusively on the cell surface, similar to the distribution of dNT (Fig. 7B). Indeed, the exclusive cell surface localization of Y6A,Y8A reflected the decrease in internalization from the cell surface. Furthermore, the substitution of a valine in the Φ position of YXXΦ motif to glutamine (V11Q) did not significantly affect either the localization or internalization of HM1.24 (Fig. 7B). Therefore, these results suggest that both Tyr residues, rather than the conserved Y-based motif, are essential for proper localization and trafficking of HM1.24. We cannot rule out that each Tyr residue also contributes to the effective trafficking of HM1.24 to some extent.

Two Tyr Residues Present in the Cytoplasmic Tail of HM1.24 Bind to α-Adaptin of AP-2 Complex—The clathrin-mediated endocytosis motifs present in cargo molecules are specifically recognized by the μ2-subunit of the AP-2 complex (43). However, the internalization signal of HM1.24 did not match the
The typical Tyr-based motif. This prompted us to speculate the involvement of adaptor or accessory proteins other than the μ2-subunit in clathrin-mediated endocytosis of HM1.24. To identify proteins interacting with HM1.24 tail, we used a NH₂-terminal cytoplasmic tail of HM1.24 as the bait in a yeast two-hybrid screen of a HeLa cell cDNA library, and found two positive clones, which encode sorting nexin 6 (SNX6) and an appendage domain of α-adaptin composed of 793–939 residues (α₇₉₃₋₉₃⁹). Interaction between HM1.24 tail and SNX6 was very weak when analyzed by the yeast two-hybrid assay (Fig. 8A), and depletion of SNX6 expression by siRNA did not change the localization and endocytosis of HM1.24 (data not shown). On the other hand, the yeast two-hybrid assay exhibited a positive interaction of α₇₉₃₋₉₃⁹ with HM1.24 tail. Mutation analyses of the HM1.24 tail apparently showed that α₇₉₃₋₉₃⁹ specifically recognizes the double Tyr motif of HM1.24 tail (Fig. 8B), consistent with the inability of Y6A,Y8A endocytosis as described above. By contrast, interaction of the HM1.24 tail with the μ2-subunit was negative in our yeast two-hybrid assay, although the cytoplasmic tail of rat HM1.24 has been shown to interact with the μ2-subunit by in vitro pulldown assay (15).

Endocytosis of HM1.24 Is Dependent on the α-Adaptin, but Not μ2-Subunit, of AP-2—The requirement of α-adaptin in HM1.24 endocytosis was further investigated by transfection of HeLa cells with siRNA directed against α-adaptin, in comparison with μ2-subunit or μ2-subunit-specific siRNA. Western blot analyses revealed that transfection of α-adaptin-, β2-adaptin-, or μ2-subunit-specific siRNA effectively reduced their respective protein expression levels by >80% of control cells (Fig. 6A). Although knockdown of each subunit did not entirely affect the expression levels of CHC, the expression levels of β2-adaptin and μ2-subunit fell to ~70% by knockdown of μ2-subunit or α-adaptin and ~80% by knockdown of β2- or α-adaptin, respectively. On the other hand, the expression levels of α-adaptin were moderately reduced to ~40% in μ2-subunit siRNA-treated cells, but not significantly in β2-adaptin siRNA-treated cells.

![FIGURE 6. Requirement of α-adaptin of AP-2 complex for HM1.24 endocytosis. A, HeLa cells were transfected with siRNAs directed to negative control (NC), μ1A-subunit, μ2-subunit, α-adaptin, β2-adaptin, CHC, or to caveolin-1. 72 h after transfection, equal amounts of cell lysates of mock- and siRNAs-treated cells were subjected to SDS-PAGE and immunoblotting using antibodies to μ1A-subunit, μ2-subunit, α-adaptin, β2-adaptin, CHC, or caveolin-1. B, negative control-, μ1A-subunit-, μ2-subunit-, α-adaptin-, β2-adaptin-, or CHC-siRNA-treated HeLa cells preincubated with HM1.24 antibody (red) and Alexa488-Tfn (green) or Rh-EGF (data not shown) for 30 min at 4 °C were incubated at 37 °C for 10 min. Non-internalized ligand was removed by washing at pH 3.0, 4 °C. Cells were then fixed, permeabilized, stained with Cy3-conjugated secondary antibody, and visualized by immunofluorescence microscopy. In caveolin-1 siRNA-treated cells, cell-internalized HM1.24 antibodies were fixed, permeabilized, and incubated with caveolin-1 antibodies. Subsequently, cells were incubated with Cy3- and Alexa488-conjugated secondary antibodies. Bars, 10 μm. C–E, quantification of the HM1.24 antibody (C), Alexa488-Tfn (D), and Rh-EGF (E) uptake assay described in B. All images were taken with identical acquisition parameters, and the amounts of internalized HM1.24 antibody, Alexa488-Tfn, and Rh-EGF are measured as expressed arbitrary units.]

15934 JOURNAL OF BIOLOGICAL CHEMISTRY

VOLUME 284 • NUMBER 23 • JUNE 5, 2009
observed a partial inhibitory effect of knockdown of the 
mechanistically differs from that of TfnR (42, 46), we also 
found that suppression of any one of the subunits in not 
reduced the internalization of Alexa488-Tfn to 
20% of con-

The knockdown of α-adaptin, β2-adaptin, or μ2-subunit reduced the internalization of Alexa488-Tfn to <20% of control cells, comparable to that observed in CHC siRNA-treated cells (Fig. 6, B and D), which can correlate with, and account for, the increased expression of TfnR on the cell surface (supple-
mental Fig. S3). In agreement with a recent report showing that the requirement of AP-2 subunit for the endocytosis of EGFR mechanistically differs from that of TfnR (42, 46), we also observed a partial inhibitory effect of knockdown of the α-adaptin, but not of β2-adaptin, or μ2-subunit, on EGFR endocytosis (Fig. 6E). In contrast to the internalization of Alexa488-Tfn, that of HM1.24 antibodies was not significantly 
affected by cells transfected with siRNA directed against 
β2-adaptin or μ2-subunit (Fig. 6, B and C). This was apparently 
different from the results reported by Rollason et al. (15). They 
showed that the inhibitory effect of μ2-subunit knockdown on 
the endocytosis of endogenous HM1.24 in HeLa cells. Interest-
ingly, the internalization of HM1.24 antibodies was specifically 
 inhibited by the transfection of α-adaptin-specific siRNA (Fig. 
6, B and C). This was further supported by the increase in the 
expression of HM1.24 on the cell surface, in α-adaptin siRNA-
treated cells, as seen in CHC-depleted cells, but not in β2-adap-
tin, or μ2-subunit siRNA-treated cells (supplemental Fig. S3). 
These results, together with the results of the yeast two-hybrid 
assay (Fig. 8, A and B), clearly suggest that α-adaptin functions 
as an adaptor protein responsible for clathrin-mediated endo-
cytosis of HM1.24, but β2-adaptin and μ2-subunit are dispensable.

**Overexpression of α-Adaptin Appendage Domain Can Recover the Endocytosis of HM1.24 in α-Adaptin-depleted Cells**—

When FLAG-tagged α93-939 was transiently expressed in HeLa cells, internalization of the HM1.24 antibody increased 
~2-fold compared with untransfected cells (Fig. 9, A and C). By 
contrast, the expression of FLAG-α93-939 did not affect the 
internalization of Alexa488-Tfn or Rh-EGF (Fig. 9, B and D); 
therefore, the results suggest that endocytosis of HM1.24 
depends on α93-939. In this context, this was somewhat sur-
prising, because a previous work showed that overexpression of the α-adaptin appendage domain inhibited Tfn uptake, prob-
ably by competing with the AP-2-dependent endocytosis mech-

![Figure 7. Analysis of endocytosis signal of HM1.24.](image_url)
anism (47). This difference probably accounts for the fact that our α793–939 lacked the binding site (including at least residues 703–756) for Eps15 (48), one of the accessory proteins involved in clathrin coat assembly on the plasma membrane. Several lines of evidence showed that Eps15 interacts with the appendage domain of α-adaptin via its DIII domain, and overexpression of this domain therefore inhibits both clathrin coat assembly and Tfn uptake (49, 50). Indeed, we found that GFP-Eps15DIII can bind to the appendage domain of α-adaptin (700–939 amino acids) containing the DIII binding site, but not to α793–939 (supplemental Fig. S5). Moreover, the overexpression of GFP-Eps15DIII revealed a significant inhibitory effect on the internalization of Cy3-Tfn, but not of the HM1.24 antibody (Fig. 9E). Consistent with recent findings (46), internalization of Rh-EGF was also not inhibited by the overexpression of GFP-Eps15DIII (Fig. 9E). We further examined whether α793–939 is necessary and sufficient for the endocytosis of HM1.24. For this purpose, FLAG-α793–939 was transiently expressed in HeLa cells depleted of the α-adaptin by transfection of α-adaptin siRNA. As shown in Fig. 10B, in cells overexpressing FLAG-α793–939 uptake of HM1.24 antibody was significantly increased, whereas both Alexa488-Tfn and Rh-EGF were hardly internalized. Consistent with the results of Fig. 9 (A and B), in cells transfected with control siRNA overexpression of FLAG-α793–939 caused increase in the internalization of HM1.24 antibody, but not Alexa488-Tfn and Rh-EGF (Fig. 10A). Because overexpression of FLAG-α793–939 in cells depleted of CHC by siRNA did not cause internalization of the HM1.24 antibody (data not shown), it is conceivable that the effect of FLAG-α793–939 on the rescue of HM1.24 endocytosis is required for clathrin. We further detected the interaction of α793–939 with CHC by the pulldown assay from HeLa cell extracts using GST-
α-adaptin-dependent HM1.24 Endocytosis

DISSCUSSION

We demonstrated that human HM1.24 is localized to lipid rafts on not only the cell surface but also TGN/recycling endosomes via the GPI anchor. In addition to the GPI anchor-deleted mutant, PI-PLC treatment of HeLa cells rendered HM1.24 Triton X-100 soluble. These results imply that the NH₂-terminal transmembrane domain of human HM1.24 is anchored outside of the lipid rafts, as previously suggested (13). Although the GPI anchor was needed to localize HM1.24 to lipid rafts, it did not significantly contribute to the steady-state localization and trafficking of HM1.24. In contrast to the results reported by Rollason et al. (15), expression of the deletion mutant of the GPI anchor and treatment of HeLa cells with PI-PLC rather enhanced the internalization of HM1.24 and redistribution of HM1.24 from the cell surface to EEA1-positive compartments, respectively (supplemental Fig. S4). We conclude therefore that the GPI anchor is hardly required for the internalization of HM1.24, rather suggesting the negative regulation of GPI in HM1.24 endocytosis.

Even after being endocytosed, HM1.24 resided in the detergent-insoluble microdomains within endosomes containing internalized Tfn. This clearly indicates that HM1.24 is internalized directly from lipid rafts without laterally moving into non-lipid rafts, from where Tfn is internalized. There is accumulating evidence that proteins localized in lipid rafts are endocytosed in a clathrin-dependent manner, which include cellular prion protein (51), the anthrax toxin receptor (52, 53), the cellular prion protein (51), and the B cell receptor (54, 55). Although lipid rafts play a critical role in the internalization of these proteins, disruption of lipid rafts by MβCD or nystatin did not cause a significant reduction of HM1.24 endocytosis, suggesting that lipid raft integrity is not a prerequisite for HM1.24 internalization. Such a different requirement of lipid rafts in clathrin-dependent endocytosis therefore may account for, and correlate to, the unique topology of HM1.24 in lipid rafts.

Deletion and site-directed mutagenesis experiments show that the cytoplasmic tail contains an essential signal to mediate not only the normal distribution, but also trafficking, of HM1.24. Although HM1.24 also has a consensus Y-based motif, YCRV, both the Tyr and Val residues did significantly function in HM1.24 endocytosis. The inability of the Y-based motif of HM1.24 to act in endocytosis may be supported by evidence that the presence of a V in the Φ position disfavors interaction with adaptor proteins (56). Rather, two Tyr residues were necessary for endocytosis of HM1.24. Notably, yeast two-hybrid screening led to identification of the appendage domain of α-adaptin as an interacting partner of the HM1.24 tail. In particular, both Tyr residues were necessary for interaction with the appendage domain of α-adaptin. Moreover, the endocytosis of HM1.24 was greatly impaired by the knockdown of α-adaptin, but not μ2-subunit and β2-adaptin. Thus, our results led to the conclusion that α-adaptin is an essential “adapter protein” that mediates the clathrin-dependent endocytosis of HM1.24.

The importance of both Tyr residues in HM1.24 trafficking has also been described in the rat counterpart (15). The results described by Rollason et al. (15) who showed the interaction of the μ2-subunit with rat HM1.24 tail and impairment of HM1.24 endocytosis in HeLa cells depleted of the μ2-subunit by siRNA, however, apparently differ from our results. These differences may be due to differences in the experimental designs, amino acid sequences of the cytoplasmic tail between rats and humans, and/or the antibodies used in the endocytosis assay, whereas we further confirmed the requirement of α-adaptin, but not μ2-subunit, for the endocytosis of HM1.24 by using either mouse monoclonal antibody against HM1.24 or rabbit polyclonal antibody against the extracellular domain of HM1.24 (data not shown), as well as humanized monoclonal antibody against HM1.24. Additionally, Rollason et al. (15) revealed that rat HM1.24 tail is recognized by not only the μ2-subunit of AP-2, but also the μ1A-subunit of AP-1, which are required for sequential trafficking of rat HM1.24, endocytosis, and early endosome-to-TGN pathways, respectively, whereas amino acid sequences within rat HM1.24 tail recognized by each μ-subunit are not determined. Our results also confirm the involvement of μ1A-subunit in the endosome-to-TGN trafficking of HM1.24 and indicate that interaction of HM1.24 tail with the μ1A-subunit is particularly mediated by Tyr-6 and Val-11 within the consensus Y-based motif (YCRV-6 also slightly contributes to the endocytosis signal), rather than the dual Y motif (supplemental Fig. S6A). Because the knockdown of μ1A altered the MPR300-positive perinuclear localization of HM1.24 to EEA1-positive early endosomes (supplemental Fig. S6B and C), the retrograde

---

**FIGURE 9. Effect of overexpression of α-adaptin appendage domain on HM1.24 endocytosis.** FLAG-α-adaptin appendage domain was transiently expressed in HeLa cells. At 24 h post-transfection, cells were incubated with HM1.24 antibody and Alexa488-Tfn (A and C) or Alexa488-Tfn and Rh-EGF (B and D) for 10 min at 37 °C, fixed, and processed for indirect immunofluorescence microscopy. Non-internalized ligand was removed by washing at pH 3.0, 4 °C. The internalized HM1.24 antibody and FLAG-α-adaptin were detected using Cy3- and Cy5-conjugated secondary antibody (A and C). C and D, quantification of the HM1.24 antibody and Alexa488-Tfn or Alexa488-Tfn and Rh-EGF uptake assay described in A and B, respectively. HM1.24 antibody, Alexa488-Tfn, or Rh-EGF uptake in randomly chosen fields of cells was quantified using NIH ImageJ software. For each field taken, all cells were outlined by hand, and the total intensity of each signal for the selected area was measured. The total intensity of HM1.24 antibody or Rh-EGF was divided by that of Alexa488-Tfn in cells transfected or untransfected FLAG-α-adaptin. All images were taken with identical acquisition parameters, and the amount of internalized HM1.24 antibody, Alexa488-Tfn, and Rh-EGF was measured as expressed arbitrary units. E, HeLa cells were transiently transfected with an expression plasmid encoding GFP-Eps15DIII (green). After 24 h, cells preincubated with Cy3-Tfn, Rh-EGF, anti-HM1.24 antibody or anti-CDS9 antibody (red) for 30 min at 4 °C were further incubated at 37 °C for 10 min and fixed. Non-internalized ligand was removed by washing at pH 3.0, 4 °C before fixation. Cells incubated with HM1.24 antibody and anti-CDS9 antibody were permeabilized, and stained with Cy3-conjugated secondary antibody. Cells were then visualized by confocal microscopy. Bars, 10 μm.
transport of HM1.24 from early endosomes to the TGN may be mediated through recognition of the Y-based motif by μ1A-subunit. Such overlapping but distinct sequence requirement by adaptor proteins has been demonstrated in the MPR300 tail: YKYSVK for AP-2 and YSKV for AP-1 (57).

The most highlighted finding in this study is that α-adaptin, especially the appendage domain, was necessary and sufficient for clathrin-mediated endocytosis of HM1.24, but not of TfnR and EGFR. Thus, the preferential requirement of α-adaptin in the endocytosis of HM1.24 clearly differs from μ2-subunit in TfnR and AP180/CALM (clathrin-assembly lymphoid myeloid leukemia protein) and Grb2 in EGFR (40, 46). The principal role of the α- and β2-adaptin appendage domain is the recruitment of a number of accessory proteins involved in the clathrin-dependent endocytic process (58). For example, the appendage domain of α-adaptin interacts with multiple accessory proteins, such as Amphiphysin, AP180, Auxilin, Eps15, Epsin, HIP, Numb, Stonin, and so on via two distinct binding sites, an NH2-terminal β-sandwich domain (side site) and a COOH-terminal mixed platform domain (top site) (59). A side site binds to WVXF-motif of accessory proteins, but a top site binds to DXF/W, FXDWF, and FXFXXL peptide motifs (59). The binding of accessory proteins with the appendage domain of α-adaptin competes with the motif domain (MD) of Eps15 containing fifteen DPF sequences that could bind to the top site of the appendage domain (59). However, in addition to no inhibitory effect of the overexpression of GFP-Eps15DIII, which contains Eps15-MD, on the internalization of HM1.24 antibody, overexpression of the appendage domain of α-adaptin lacked most of the side site in cells depleted of α-adaptin that could rescue the internalization of HM1.24 antibody, but not Tfn and EGF. These results apparently indicate that interaction of the dual Tyr motif of HM1.24 with the appendage domain of α-adaptin occurs at a different site from that with various accessory proteins for binding to the top site of the appendage domain. The dual Tyr motif of HM1.24 may thus function as a new binding motif for the appendage domain of α-adaptin. Unfortunately, we did not detect a direct interaction between the cytoplasmic tail of HM1.24 and the appendage domain of α-adaptin by in vitro pulldown assay. This may reflect the weak interaction between these molecules. Alternatively, the presence of other adaptor-like proteins that mediate the stable interaction of HM1.24 tail with the appendage domain of α-adaptin cannot be completely ruled out. In this context, the appendage domain of α-adaptin might function in the endocytosis of HM1.24 from lipid rafts as a scaffold protein that recruits the accessory protein required for clathrin-coated vesicle formation.

It was recently reported that HM1.24 prevents HIV-1 release from the surface of cells, which is counteracted by the HIV-1 accessory protein, Vpu (11, 12). Therefore, in the absence of Vpu, massive attachment of the assembled HIV-1 particles at the cell surface occurs in HM1.24-expressing cells, such as HeLa cells. Colocalization of HM1.24 with the HIV-1 structural protein Gag, not only at the plasma membrane, but also in endosomes, has suggested that HM1.24 traps virus on and within infected cells. Although it is not known whether HM1.24 is directly involved in the endocytosis of these proteins, the expression of HM1.24 induced intracellular accumulation of Gag in the absence of Vpu expression (11). Moreover, consistent with the lipid raft localization of HM1.24, many viruses, including HIV-1, bud from lipid raft domains and therefore have very similar lipid composition to rafts of host cell membranes (60). In this case, partitioning of HM1.24 into lipid raft domains on the cell surface seems to help promote encounters with assembling HIV-1 particles (11), because removal of the GPI anchor completely abolished the ability of tetherin to inhibit Vpu-deleted HIV-1 virion release. Notably, the expression of Eps15-DIII did not block the endosomal accumulation of either Gag or HIV envelope glycoprotein in HeLa cells expressing vpu-negative virus (61). These results, coupled with the data presented in this study, may further strengthen the hypothesis that HM1.24 is internalized by a novel endocytic mechanism, which is independent of the typical AP-2 adaptor complex.

It is also interesting to note that the appendage domain of α-adaptin can bind to CHC. Generally, the binding of AP-2 complex with clathrin has been considered to be mediated by the β2-adaptin. Clathrin binding regions of β2-adaptin have been mapped to the flexible hinge domain having the consensus sequence L(L/I)(D/E/N)(L/F)(D/E), referred to as the “clathrin box” (62). The hinge and appendage domains of β2-adaptin stimulate the assembly of clathrin lattices in vitro (63). A similar clathrin box sequence, LLRLE, is present at the position of 903–907 of the appendage domain of α-adaptin. Although we have not determined the involvement of these sequences in clathrin binding, there is a report that α-adaptin containing hinge and appendage domains can bind clathrin but is far weaker than the hinge and appendage domains of β2-adaptin (63). Based on these lines of evidence, it is conceivable that, in the absence of other adaptor proteins, α-adaptin can recognize the dual Tyr residue of the HM1.24 tail and can recruit clathrin to ensure the formation of clathrin-coated vesicles. This conclusion may be supported from the result that, even in cells depleted of μ2-subunit, clathrin-coated vesicles are still formed at the plasma membrane (42). In addition to clathrin-binding capacity, β2-adaptin can directly bind with the dileucine-based motif via the trunk domain (64, 65) or the appendage domain (5). The appendage domains of both α-adaptin and β2-adaptin have overlapping binding to accessory proteins (5). Therefore, the appendage domains of both α- and β2-adaptins may

FIGURE 10. Clathrin- and α-adaptin appendage domain-dependent endocytosis of HM1.24. Forty-eight hours after transfection of siRNA directed to negative control (A) or α-adaptin (B), HeLa cells were transiently transfected with an expression plasmid encoding the FLAG-793–939. Cells then internalized the FLAG-793–939-HM1.24 antibody and Alexa488-Tfn or Alexa488-Tfn and Rh-EGF, as described in Fig. 9 (A and B). Non-internalized ligand was removed by washing at pH 3.0, 4°C before fixation. C, HeLa cell lysates were precipitated with GST or GST-793–939. Precipitates and aliquots of cell lysates were subjected to SDS-PAGE and immunoblotting using antibody to CHC. D, HeLa cells were transiently transfected with an expression plasmid encoding FLAG-793–939. Forty-eight hours after transfection, cell lysates were immunoprecipitated with the antibody to FLAG, and immunoprecipitates were subjected to SDS-PAGE and immunoblotting using antibodies to CHC, or FLAG. IB, immunoblotting.
also function in direct recognition of cargo in the clathrin-dependent process, as well as the recruitment of accessory proteins. Although further investigations are needed to confirm how α-adaptin regulates the endocytosis of HM1.24, our results in this study provide new insight into the molecular mechanism of clathrin-mediated endocytosis from lipid rafts.

Acknowledgment—We thank Dr. J. S. Bonifacino for providing human µ1A- and µ2-subunit constructs.

REFERENCES

1. Ozaki, S., Kosaka, M., Wakatsuki, S., Abe, M., Koishihara, Y., and Matsumoto, T. (1997) Blood 90, 3179–3186
2. Ohtomo, T., Sugamata, Y., Ozaki, Y., Ono, K., Yoshimura, Y., Kawai, S., Koishihara, Y., Ozaki, S., Kosaka, M., Hirano, T., and Tsuichii, M. (1999) Biochem. Biophys. Res. Commun. 258, 583–591
3. Ishikawa, J., Kaisho, T., Tomizawa, H., Lee, B. O., Kobune, Y., Inazaka, J., Oritani, K., Itoh, M., Ochi, T., and Ishihara, K. (1995) Genomics 26, 527–534
4. Goto, T., Kennel, S. I., Abe, M., Takishita, M., Kosaka, M., Solomon, A., and Saito, S. (1994) Blood 84, 1922–1930
5. Schmid, E. M., Ford, M. G., Burtney, A., Praefcke, G. J., Peak-Cheek, S. Y., Mills, I. G., Benmerah, A., and McMahon, H. T. (2006) PLoS Biol. 4, e262
6. Vidal-Laliena, M., Romero, X., March, S., Requena, V., Petriz, J., and Engel, P. (2005) Cell. Immunol. 236, 6–16
7. Walter-Yohrling, I., Cao, X., Callahan, M., Weber, W., Morgenbesser, S., Madden, S. L., Wang, C., and Teicher, B. A. (2003) Cancer Res. 63, 8939–8947
8. Grützmann, R., Boriis, H., Ammerpohl, O., Lüttges, J., Kalthoff, H., Schackert, H. K., Klöppel, G., Saeger, H. D., and Pilsarky, C. (2005) Oncogene 24, 5079–5088
9. Capurso, G., Latitmore, S., Cnorgorac-Jurcivic, T., Panzuto, F., Milione, M., Bhatia, V., Campanini, N., Swift, S. M., Bordi, C., Delle Fave, G., and Lemoine, N. R. (2006) Endoc. Relat. Cancer 13, 541–558
10. Blausis, A. L., Giuriato, E., Cell, M., Schreiber, R. D., Shaw, A. S., and Colonna, M. (2006) J. Immunol. 177, 3260–3265
11. Neil, S. J., Zang, T., and Bieniasz, P. D. (2008) Nature 451, 425–430
12. Van Damme, N., Goff, D., Katsura, C., Jorgenson, R. L., Mitchell, R., Johnson, M. C., Stephens, E. B., and Guatelli, J. (2008) Cell Host Microbe 3, 245–252
13. Kupzig, S., Korolchuk, V., Rollason, R., Sugden, A., Wilde, A., and Banting, G. (2003) Traffic 4, 694–709
14. Li, X., Kaloyanova, D., van Eijk, M., Zizioli, D., Lausmann, S., Eskelinen, E. L., Hamann, J., Saftig, P., von Figure, K. and Schu, P. (2000) EMBO J. 19, 2193–2203
15. Peden, A. A., Rudge, R. E., Lui, W. W., and Robinson, M. S. (2002) J. Cell Biol. 156, 327–336
16. Johannesen, L. E., Pedersen, N. M., Pedersen, K. W., Madhus, I. H., and Stang, E. (2006) Mol. Cell. Biol. 26, 389–401
17. Owen, D. J., Vallis, Y., Noble, M. E., Hunter, J. B., Dafforn, T. R., Evans, P. R., and McMahon, H. T. (1999) Cell 97, 805–815
18. Benmerah, A., Bègue, B., Dautey-Varsat, A., and Cerf-Bensussan, N. (1996) J. Biol. Chem. 271, 12111–12116
19. Benmerah, A., Lamaze, C., Bègue, B., Schmid, S. L., Dautey-Varsat, A., and Cerf-Bensussan, N. (1998) J. Cell Biol. 140, 1055–1062
20. Benmerah, A., Poupon, V., Cerf-Bensussan, N., and Dautey-Varsat, A. (2000) J. Biol. Chem. 275, 3288–3295
21. Taylor, D. R., Watt, N. T., Perera, W. S., and Hooper, N. M. (2005) J. Cell Biol. 118, 5141–5153
22. Abram, L., Liu, S., Cosson, P., Leppha, S. H., and van der Goot, F. G. (2003) J. Cell Biol. 160, 321–328
23. Abram, L., Leppha, S. H., and van der Goot, F. G. (2006) J. Cell Biol. 172, 309–320
24. Stoddard, A., Dykstra, M. L., Brown, B. K., Song, W., Pierce, S. K., and Brodsky, F. M. (2002) Immunity 17, 451–462
25. Stoddard, A., Jackson, A. P., and Brodsky, F. M. (2005) Mol. Cell. Biol. 25, 2339–2348
26. Ohno, H., Aguilar, R. C., Jey, D., Taura, D., Saito, T., and Bonifacino, J. S. (1998) J. Biol. Chem. 273, 25915–25921
27. Canfield, W. M., Johnson, K. F., Ye, R. D., Gregory, W., and Kornfeld, S. (1991) J. Biol. Chem. 266, 5682–5688
28. Schmid, E. M., and McMahon, H. T. (2007) Nature 448, 883–888
29. Praefcke, G. J., Ford, M. G., Schmid, E. M., Olesen, L. E., Gallop, J. L., Peak-Cheek, S. Y., Vallis, Y., Babu, M. M., Mills, I. G., and McMahon, H. T.
60. Brügger, B., Glass, B., Haberkant, P., Leibrecht, I., Wieland, F. T., and Kräusslich, H. G. (2006) *Proc. Natl. Acad. Sci. U. S. A.* **103**, 2641–2646
61. Van Damme, N., and Guatelli, J. (2008) *Cell. Microbiol.* **10**, 1040–1057
62. Dell’Angelica, E. C., Klumperman, J., Stoorvogel, W., and Bonifacino, J. S. (1998) *Science* **280**, 431–434
63. Shih, W., Gallusser, A., and Kirchhausen, T. (1995) *J. Biol. Chem.* **270**, 31083–31090
64. Rapoport, I., Chen, Y. C., Cupers, P., Shoelson, S. E., and Kirchhausen, T. (1998) *EMBO J.* **17**, 2148–2155
65. Yao, D., Ehrlich, M., Henis, Y. I., and Leof, E. B. (2002) *Mol. Biol. Cell* **13**, 4001–4012