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Human Yip1A specifies the localization of Yif1 to the Golgi apparatus

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Abstract

Yip1p and Yif1p are essential for transport from ER to Golgi stack during the early secretory pathway in budding yeast. Here, we report the identification and characterization of human Yif1. Sequence analysis revealed that human Yif1 (HsYif1), like most of the other YIP1 protein family members, contains multiple transmembrane segments. Double immunofluorescence study revealed co-distribution of HsYif1 with Golgi marker such as GS27. To delineate the function of HsYif1, we conducted a yeast two-hybrid assay and identified an interaction between human HsYif1 and HsYip1A, a homolog of yeast Yip1. In addition, our immunoprecipitation pull-down assay validates the interaction between HsYif1 and HsYip1A. Moreover, our immunofluorescence study demonstrates the co-distribution of HsYif1 and HsYip1A. Significantly, over-expression of mutant HsYip1A-lacked cytosolic region disrupts the localization of HsYif1 to the Golgi, suggesting that HsYip1A specifies the localization of HsYif1 to the Golgi. Therefore, we conclude that human Yip1A interacts with and determines the localization of HsYif1 to the Golgi apparatus.

Keywords: Yif1; Yip1A; Golgi; ER

Intracellular transport between the endoplasmic reticulum (ER) and Golgi is bidirectional and is mediated by coat protein complexes (COPI and COPII). COPII is involved in anterograde transport from the ER while retrograde transport of recycled proteins from Golgi and pre-Golgi compartments back to the ER relies on COPI. The specificity and directionality of membrane traffic rely on complex set of proteins whose general structures and functions are postulated to be conserved from yeast to human (e.g., [1]).

Yip1p was identified as Ypt1p and Ypt31p interacting protein in a yeast two-hybrid screen [2], and was required for transport through the early secretory pathway and function during COPII vesicle biogenesis [3]. Yip1p interaction with Rab proteins requires double geranyl–geranyl groups which in turn are required for Rab proteins to correctly localize to their characteristic organelle membrane [4]. Genetic studies indicate that Rab-GDI and a critical subset of Rab proteins are crucial for the action of Yip1p [5]. Yif1p was later identified as Yip1p interacting factor in a two-hybrid screen [6] and it is enriched in COPII vesicles [7]. Both Yip1p and Yif1p are essentially Golgi-localized proteins and bind to Rab GTPases in yeast [2,6]. Yip1p forms a heteromeric complex with Yif1p [5,7] and this Yip1p–Yif1p complex is required for the membrane fusion competence of ER-derived vesicles and binds the ER to Golgi SNAREs Bos1p and Sec22p [8]. Yos1p was recently discovered as a novel subunit of the Yip1p–Yif1p complex [9]. Yos1p associates with Yip1p and Yif1p, and is required for transport between the ER and the Golgi complex [9]. Another study showed that the N-terminal cytoplasmic domain of Yip1p and Yif1p mediates their

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interactions with Phox homology (PX) domain of Grd19p, Vam7p, Vps5p, Vps17p, and Ypt35p [10].

Yip1p is a prototype for YIP protein family [11], which contains: (1) a common topology with a cytosolic N terminus, luminal C terminus, and multiple transmembrane-spanning domains; (2) ability binding to Rab GTPases; (3) capacity interacting with each other. Yip2p (Yop1p) [12], Yip3p [13], Yip4p [11], Yip5p [11], Yif1p, and their mammalian homologs also belong to YIP1 family. There are many members of the YIP1 family in mammals [14], but only Yip1A and PRA1 are well studied. Mammalian Yip1A (mouse and human), a homolog of yeast Yip1p, is enriched at the ER exit sites (ERES), binds to the Sec23–Sec24 complex, and is believed to be involved in ER–Golgi transport [15]. PRA1, homolog of yeast Yip3p, is a dual prenylated Rab GTPase interacting with VAMP2 [16]. A DXEE motif in the COOH-terminal domain is essential for targeting PRA1 to the Golgi [17].

Recently, it was reported that human Yif1 was a member of cycling proteins localized to ER–Golgi intermediate compartment (ERGIC) [18], but its function remains elusive. In this study, we demonstrate that HsYif1 is localized to the Golgi and interacts with HsYip1A. Furthermore, our study demonstrates the biochemical interaction between HsYip1A and HsYif1 and the requirement of HsYip1A for HsYif1 localization to Golgi.

Materials and methods

Antibodies. Mouse monoclonal antibody against 58 kDa Golgi protein was from Abcam; mouse monoclonal antibody against the Golgi marker protein GS27 was from BD Transduction Lab (San Jose, CA); FLAG antibody (M2) was from Sigma; HA antibody was from Cell Signaling Technology.

Clones and constructs. Full-length human Yif1 and Yip1A genes were generated by PCR from a human Testis cDNA library (Clontech) with related primers and cloned into TA cloning vector (Takara Biotechnology, Dalian, China) and completely sequenced. GFP-tagged full-length HsYif1 and HsYip1A, N terminus of HsYif1 (1–138aa), and C terminus of HsYif1 (139–293aa) and HsYip1A (124–257aa) were constructed by inserting in-frame the PCR products into pEGFP-C1 or C2 vector (Clontech) with related restriction enzyme digestion. FLAG-tagged HsYif1, HsYip1A and HA-tagged HsYip1A cDNA were cloned by inserting the PCR product into the pcDNA3 vector (Invitrogen) with *Eco*RI and *Xho*I digestion. All plasmids were confirmed by sequencing.

Cell culture, transfection, and immunofluorescence microscopy. HeLa and 293T cells (American Type Culture Collection, Manassas, VA) were cultivated as subconfluent monolayers in Dulbecco's modified Eagle's medium (Invitrogen) with 10% fetal bovine serum (HyClone, UT) and 100 U/ml penicillin plus 100 μg/ml streptomycin at 37 °C with 10% CO_2. Transfections were performed using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. For immunofluorescence, cells were seeded onto sterile, acid-treated 18-mm coverslips in 6-well plates (Corning Glass). Twenty-four hours after transfection, HeLa cells were washed twice with PBSCM (PBS with 1 mM $CaCl_2$, 1 mM $MgCl_2$), and then fixed in 3.6% paraformaldehyde. Fixed cells were then permeabilized with 0.2% saponin (Sigma) and incubated with primary antibodies against 58 kDa Golgi protein, the Golgi marker protein GS27, and FLAG, followed by incubation with the rhodamine-conjugated secondary antibody. DNA was stained with DAPI (Sigma). Fluorescence labeling was visualized using a Zeiss Axiovert-200 fluorescence microscope, and images were collected and analyzed with Image-5 (Carl Zeiss, Germany).

Immunoprecipitation assay and Western blot. FLAG-HsYif1 and HA-HsYip1A cDNA constructs were co-transfected into 293 T cells while Golgi enriched membranes (Golgi extracts) were prepared 30 h after transfection as described previously [19]. Immunoprecipitation was carried out as described previously [20], FLAG antibody (5–10 μg) bound to protein A/G beads (Pierce) was incubated for 4 h with 500 μg of Golgi extracts in immunoprecipitation buffer (20 mM Hepes, pH 7.3, 100 mM KCl, 1 mM DTT, 5 mM EDTA, 0.2 mM ATP, 1% Triton X-100, and protease inhibitor) at 4 °C. Beads were then washed twice with buffer A (identical to immunoprecipitation buffer except that it contains 0.5% Triton X-100) and three times with buffer B (identical to buffer A except that it contains 0.2% Triton X-100) before being resuspended in SDS sample buffer. Samples were subjected to SDS–PAGE on 6–16% gradient gel and transferred onto nitrocellulose membrane. Proteins were probed by appropriate primary and secondary antibodies and detected using ECL (Pierce). The band intensity was then scanned using a PhosphorImager (Amersham Biosciences).

Yeast two-hybrid (Y2H) assay. ORF sequences of HsYif1 and HsYip1A were subcloned into pGADT7 for "bait" and pGBKT7 for "fish" constructs, and transformed into the yeast strain AH109 containing the reporter genes *his3* and *lacZ*. Double transformants were plated on selective media and incubated for 2–3 days at 30 °C. Quantitative measurements of HsYif1–HsYip1A interaction, judged by the β-galactosidase activity, were performed essentially as described in the manufacturer's manual.

Results and discussion

Identification of human Yif1, an ortholog of yeast Yif1p

Yeast Yif1p is an interacting factor of Yip1p [6] and human Yif1 was found as a cycling protein purified from ERGIC in HepG2 cell line [18]. To study the function of human Yif1, we cloned it according to the sequence available in the database. Bioinformatics analysis reveals that Yif1 is well conserved across species, possessing homologs in the very distant species, e.g., *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Caenorhabditis elegans*, *Drosophila melanogaster*, *Mus musculus*, and *Homo sapiens* (Fig. 1A). Hydrophobicity plotting [21] shows that HsYif1 contains four to five putative transmembrane domains (TMD) located at the COOH-terminal (Fig. 1B). Thus, we concluded that HsYif1 is an integral membrane protein.

HsYif1 localizes to the Golgi

To investigate the subcellular location of HsYif1, we first expressed GFP-tagged HsYif1 in HeLa cells. Indirect immunofluorescence revealed that GFP-tagged HsYif1 is localized to membrane structures and is mainly concentrated at the juxtanuclear region (data not shown). To test whether HsYif1 is a Golgi-localized pro-

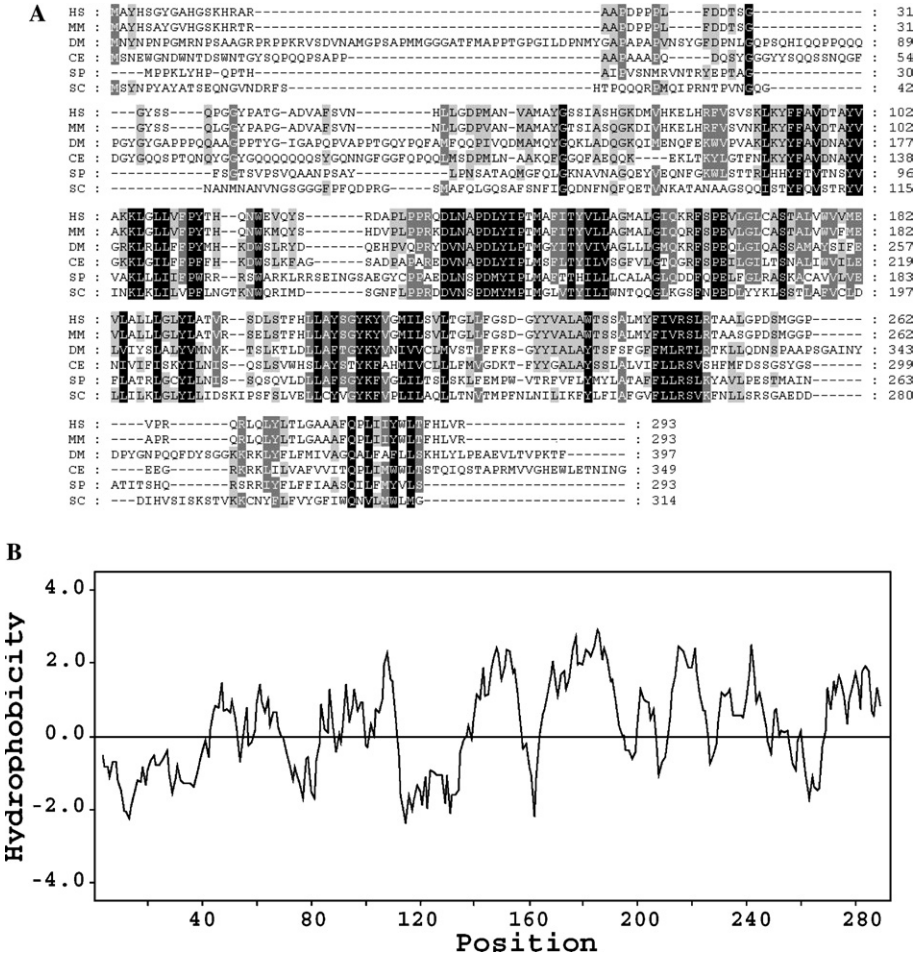

Fig. 1. Human integral membrane protein Yif1 is an ortholog of yeast Yif1p. (A) Sequence comparison of Yif1 from different species. The amino acid sequence of Yif1 from *H. sapiens* (HS) 54TM (Accession No. AAD01206) is aligned with those of related proteins from *M. musculus* (MM) Yif1 (Accession No. AAH11117), *D. melanogaster* (DM) CG5484-PA (Accession No. AAF56617), *C. elegans* (CE) F57A8.2 (Accession No. CAA94831), *S. pombe* (SP) SPBC25H2.06c (DDBJ/EMBL/GenBank Accession No. CAB08782), and *S. cerevisiae* (SC) Yif1p (Accession No. NP_014136). Alignments were generated using the ClustalW program. Invariant residues are shaded in black, while regions in which conserved substitutions have been observed are shaded in grey. (B) Hydrophobicity plot of the Yif1 amino acid sequence as described in [21].

tein, we carried out double immunofluorescence in GFP-HsYif1-expressing HeLa cells using two Golgi marker proteins, 58 kDa Golgi protein and GS27, respectively. As shown in Figs. 2A (b, f), labeling of 58 kDa Golgi protein and GS27 gave a typical delineation of the *cis*-Golgi apparatus. Examination of GFP-HsYif1-transfected cells reveals that HsYif1 is co-localized with *cis*-Golgi marker 58 kDa Golgi protein and GS27 as the two images merged (Figs. 2A (d, h)). 58 kDa Golgi protein is a formiminotransferase cyclodeaminase (FTCD), an enzyme that catalyzes two consecutive steps in the modification of tetrahydrofolate to 5,10-methenyl tetrahydrofolate [22,23]. FTCD appears to be a dynamic component of the Golgi, and a proportion of FTCD molecules cycles between the Golgi and earlier compartments of the secretory pathway [23]. GS27 participated in protein movement from the medial- to *trans*-Golgi and the *trans*-Golgi network [24]. Co-localization of Yif1 with 58 kDa Golgi protein and GS27 suggested that HsYif1 is a Golgi protein.

To investigate the subcellular localization of HsYif1 during cell cycle, GFP-HsYif1-expressing HeLa cells were labeled with 58 kDa Golgi protein as a Golgi marker. As shown in Fig. 2B, examination of GFP-HsYif1 distribution reveals a typical Golgi vesicular labeling concentrated at the juxtanuclear region in interphase cells (Figs. 2B (a–d)). The Golgi apparatus undergoes dynamic fragmentation as cells enter into mitosis and then reassembly before cells exit from the telophase. Examination of HsYif1 distribution in mitotic cells reveals a diffuse staining throughout the cytoplasm in metaphase cells (Figs. 2B (e–h)) as the dissolution of Golgi apparatus. The scattered Golgi-derived vesicles become reassembled in anaphase (Figs. 2B (i–l)) as the mitotic kinase activity declines and the coalescence of Golgi fragments enriched in GFP-tagged HsYif1 was

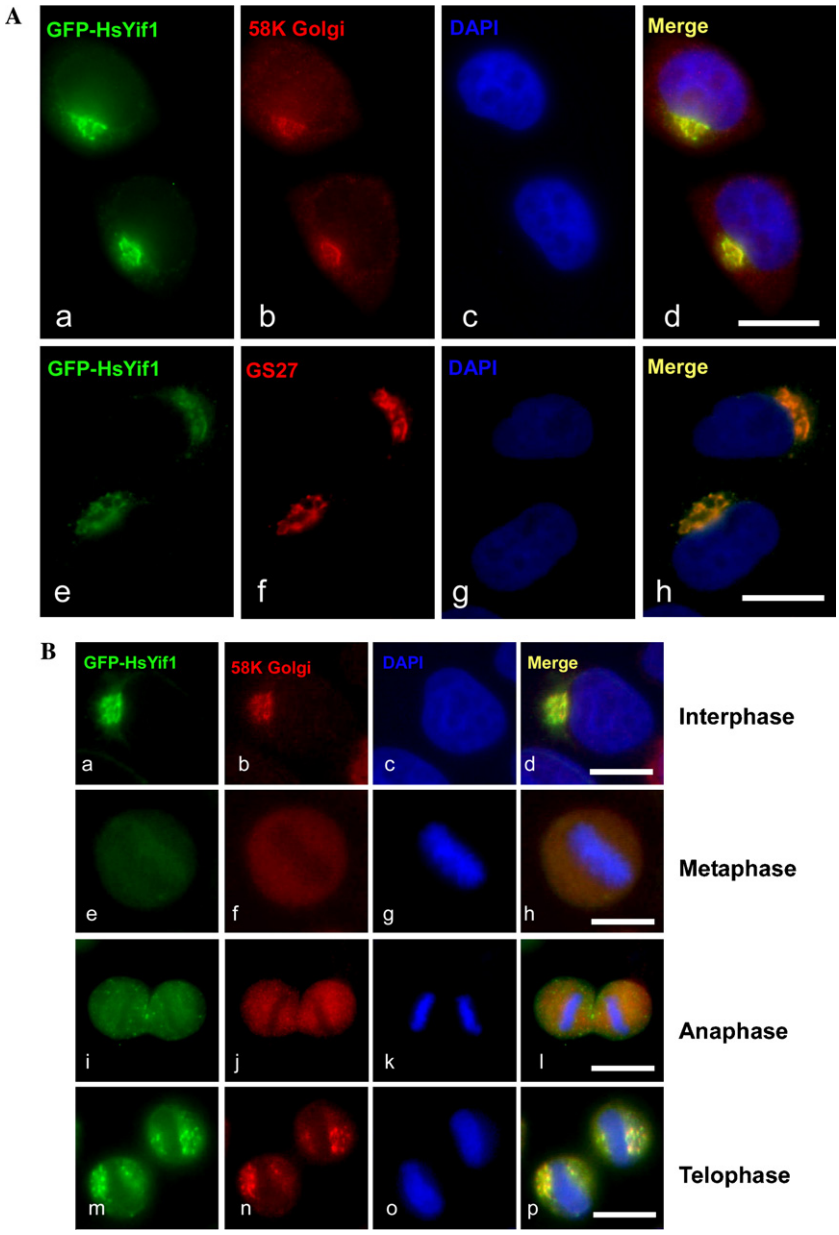

Fig. 2. Human Yif1 is a Golgi-localized protein. HeLa cells were transfected with N-terminal GFP-tagged full-length HsYif1 and stained with anti-58 kDa Golgi protein antibody or anti-GS27 antibody, and DAPI. The rhodamine-conjugated anti-mouse IgG was used as a secondary antibody. Triply stained optical images were collected as GFP-HsYif1 (green), 58 kDa Golgi protein or GS27 (red), DAPI (blue), and their merged images. (A) Co-localization of HsYif1 with Golgi 58 kDa protein and GS27. (B) Subcellular localization of HsYif1 during cell cycle. Bars, 10 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

observed in telophase cells (Figs. 2B (m–p)). Thus, HsYif1 undergoes reversible disassembly following the Golgi complex during cell cycle.

Both N and C termini of HsYif1 are essential for its Golgi localization

To delineate the molecular basis underlying Golgi anchoring of HsYif1, we generated two GFP-tagged deletion mutants. One is HsYif1N (1–138aa), which is hydrophilic region of HsYif1, and another is HsYif1C (139–293aa), which is hydrophobic domain containing several TMD. We then transiently expressed the deletion constructs in HeLa cells for immunofluorescence analysis. Full-length GFP-HsYif1 is localized to Golgi as we revealed in an earlier study (Fig. 2A). However, expression of the hydrophilic region of HsYif1 (i.e., HsYif1N) was diffused throughout the cell as shown in Fig. 3A. Surprisingly, the HsYif1C, despite possession of TMD, is no longer localized to the Golgi apparatus but instead to ER-like compartment (Fig. 3E). Neither HsYif1N nor HsYif1C localized to Golgi when compared with

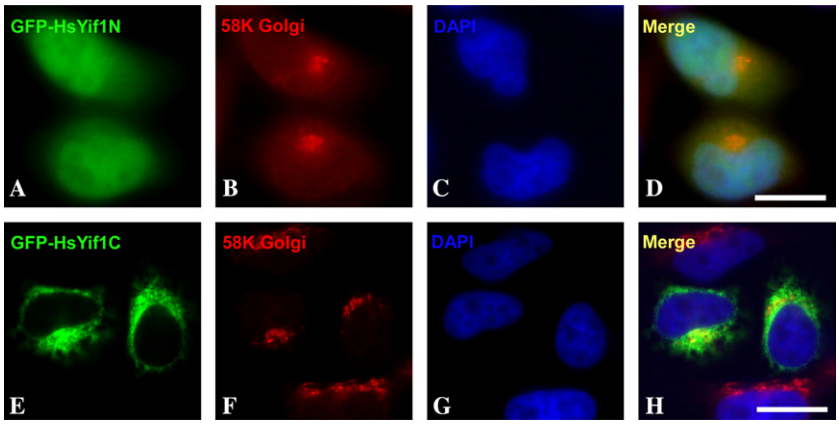

Fig. 3. Both N and C termini of HsYif1 are important for its Golgi localization. HeLa cells were transfected with GFP-tagged N terminus of HsYif1 protein (amino acids [aa] 1–138) and C terminus of HsYif1 protein (amino acids [aa] 139–293). Cells were then fixed, permeabilized, and stained with anti-58 kDa Golgi protein antibody. Triply stained optical images were collected as GFP-HsYif1N or GFP-HsYif1C (green), 58 kDa Golgi protein (red), DAPI (blue), and their merged images. Bars, 10 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

Golgi marker 58 kDa Golgi protein. Thus, we conclude that both N and C termini are required to anchor HsYif1 to Golgi.

HsYif1 interacts with HsYip1A

In yeast, Yif1p interacts with Yip1p [6]. To test whether such an interaction is conserved in human, we conducted a yeast two-hybrid assay using HsYif1 and HsYip1A as reciprocal bait and prey as illustrated in Fig. 4A. Verifying no auto-activity (data not shown), we co-transformed pGBKT7/HsYif1 with pADT7/HsYip1A and pGBKT7/HsYip1A with pADT7/HsYif1 into yeast AH109, respectively. Both types of transformation grew on nutrient selective media (positive and negative control not shown), indicated a positive interaction between HsYif1 and HsYip1A.

To validate the interaction between HsYif1 and HsYip1A, observed in our yeast two-hybrid assay, we carried out immunoprecipitation assay. FLAG-HsYif1

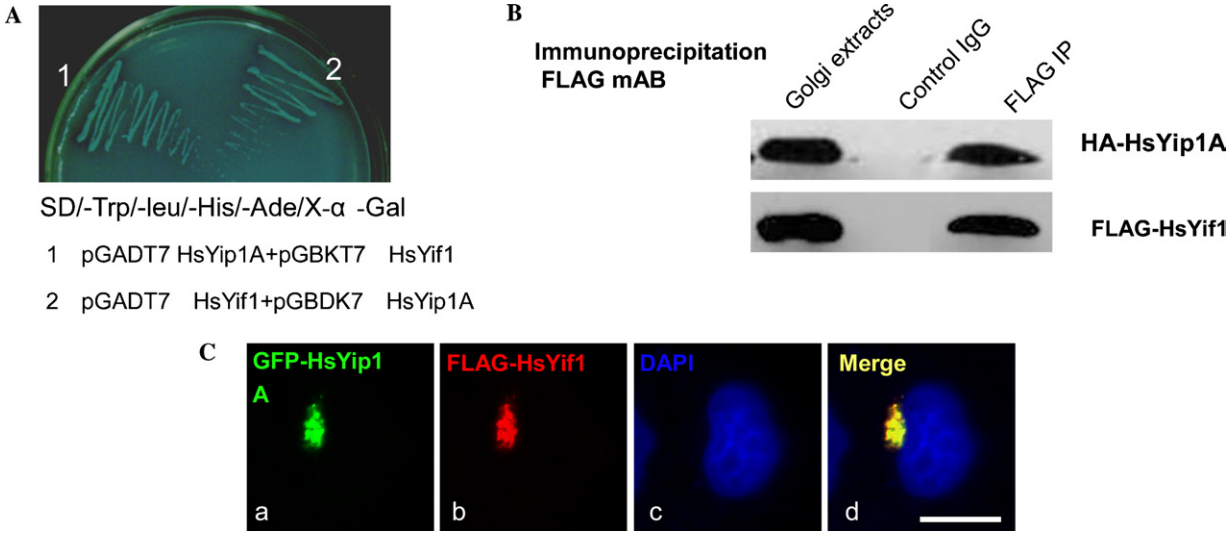

Fig. 4. HsYif1 interacts with HsYip1A. (A) Identification of HsYif1–HsYip1A interaction in yeast two-hybrid assay. Yeast cells were co-transformed with full-length HsYif1 bait or prey and full-length HsYip1A prey or bait. This is an example of selected cells on supplemented minimal plates lacking uracil, tryptophan, leucine, and histidine. (B) HsYif1 interacts with HsYip1a in vivo. FLAG-HsYif1 and HA-HsYip1A co-expressing 293T cells were harvested, FLAG antibody (5–10 μg) bound to protein A–Sepharose beads were incubated 4 h with 500 μg of Golgi extracts in immunoprecipitation buffer. Proteins from Golgi extracts and FLAG immunoprecipitates were analyzed by SDS–polyacrylamide gel electrophoresis and immunoblotting using FLAG antibody, HA antibody, respectively (Materials and methods). (C) Co-localization of HsYif1 with HsYip1A to the Golgi of HeLa cells. FLAG-HsYif1 and GFP-HsYip1A co-transfected HeLa cells were fixed, permeabilized, and stained with anti-FLAG antibody. Triply stained optical images were collected as GFP-HsYip1A (green), HsYif1 (red), DAPI (blue), and their merged images. Bar, 10 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

and HA-HsYip1A were co-transfected in 293T cells and immunoprecipitation was performed using anti-FLAG antibodies. As shown in Fig. 4B, Western blot confirmed that HA-HsYip1A was pull down by FLAG-tagged HsYif1. Neither HsYif1 nor HsYip1A was pull down by control IgG. Thus, we conclude that the interaction between HsYif1 and HsYip1A is specific and direct.

Previous studies demonstrated that HsYip1A is enriched in the ERES [15] and HsYif1 is primarily localized to Golgi (Fig. 2A) and ERGIC [18]. The finding of biochemical interaction between HsYif1 and HsYip1A propelled us to test whether HsYif1 is co-localized and to which compartments. To this end, we co-transfected HeLa cells with FLAG-HsYif1 and GFP-HsYip1A. Immunofluorescence labeling of HsYif1 using FLAG antibody followed by a rhodamine-conjugated secondary antibody clearly marks the Golgi appearance in interphase cells (Fig. 4C (a)). GFP-HsYip1A of the same cells revealed a similar pattern seen in HsYif1 staining as show in Fig. 4C (b). It became readily evident when HsYif1 labeling is merged with HsYip1A staining as two colors are almost superimposed (Fig. 4C (d)). Thus, HsYif1 is co-localized with HsYip1A to the Golgi. It is reported that HsYip1A localizes to the ERES and involves in ER to Golgi transport [15]. Considering the adjacency of ERES, ERGIC, and Golgi, it is not surprise that these two recycling proteins co-localize to the Golgi. Given the biochemical interaction between HsYif1 and HsYip1A, we conclude that HsYif1 interacts with HsYip1A in the Golgi compartment of human cells.

Golgi localization of HsYif1 is dependent on HsYip1A

Since HsYif1 interacts with HsYip1A and forms a complex, we ask whether any of these proteins plays any essential roles in localization and/or docking of the other protein to the Golgi. To this end, we used a set of GFP-tagged HsYif1 and HsYip1A deletion mutants, e.g., HsYif1N, HsYif1C, HsYip1AN (1–123aa), and HsYip1AC (124–257aa). These mutants were co-transfected in HeLa cells in different combinations like FLAG-HsYif1 with GFP-HsYip1AN or GFP-HsYip1AC, FLAG-HsYip1A with GFP-HsYif1N or GFP-HsYif1C. Immunofluorescence staining showed that both GFP-HsYif1N and GFP-HsYif1C did not affect the localization of FLAG-HsYip1A, and GFP-HsYip1AN did not affect the localization of FLAG-HsYif1 (data not shown). Conversely GFP-HsYip1AC changed the localization of FLAG-HsYif1, as show in Fig. 5, HsYif1 localized to ER-like GFP-HsYip1AC. GFP-HsYip1AC acts as dominant negative mutant, affecting the endogenous HsYip1A localization and disrupts HsYif1 Golgi localization. Thus HsYip1A is essential for the localization of HsYif1 to the Golgi and the TMD of HsYip1A determined HsYif1's Golgi localization.

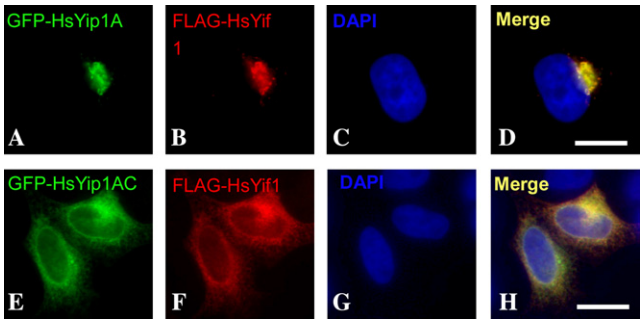

Fig. 5. HsYip1A specifies the Golgi localization of HsYif1. Full-length GFP-HsYip1A or GFP-HsYip1AC with FLAG-HsYif1 co-transfected HeLa cells were fixed, permeabilized, and stained with anti-FLAG antibody. Triply stained optical images were collected as GFP-HsYip1A or GFP-HsYip1AC (green), HsYif1 (red), DAPI (blue), and their merged images. Bars, 10 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

Yip1A binds to the Sec23–Sec24 COPII subcomplex through its N terminus [15] and over-expression of the N terminus of Yip1A induces breakdown of the Golgi apparatus and inhibits VSVG transport [15], while human Yif1 is a cycling protein of ERGIC [18]. Combined with the observation that HsYif1–HsYip1A forms a complex (this study), we propose that HsYif1–HsYip1A complex binds to the Sec23–Sec24 COPII subcomplex through HsYip1A, and is involved in ER to Golgi transport similar to that of yeast Yif1p–Yip1p complex, and HsYip1A is an important regulator of HsYif1–HsYip1A complex. While the molecular function of HsYif1 underlying ER to Golgi transport remains to be delineated, our study supports a notion that the vesicular transport machinery is conserved between yeast and human.

Acknowledgments

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