Hypothetical protein KIAA0079 is a mammalian homologue of yeast Sec24p

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Abstract The Sec23p-Sec24p complex is a component of COPII-coated vesicles that mediate protein transport from the endoplasmic reticulum in yeast. The mammalian hypothetical protein KIAA0079 (KIAA0079p) exhibits sequence similarity to yeast Sec24p. KIAA0079p was co-eluted with mammalian Sec23p on gel filtration. In vitro binding experiments revealed that the C-terminal region of KIAA0079p binds to the N-terminal region of mammalian Sec23p. Overexpression of KIAA0079p caused a defect in protein export from the endoplasmic reticulum. These results support the idea that KIAA0079p is a functional homologue of yeast Sec24p.

Key words: Sec23p; Sec24p; Coat protein II; Endoplasmic reticulum; Vesicular transport

1. Introduction

Genetic and biochemical analyses of *Saccharomyces cerevisiae* revealed that COPII-coated vesicles mediate protein transport from the endoplasmic reticulum (ER) to the Golgi apparatus [1,2]. COPII consists of a small GTP binding protein, Sar1p [3], and two protein complexes, Sec23p-Sec24p [4,5] and Sec13p-Sec31p [6,7]. These proteins are required and sufficient for the formation of COPII vesicles not only from isolated ER membranes [8] but also from liposomes [9]. In addition to the role of vesicle formation, Sar1p and the Sec23p-Sec24p complex play a role in selecting cargo proteins [10], although its molecular mechanism is not fully understood.

Mammalian counterparts of COPII except for Sec24p and Sec31p were identified [11–15], and their involvement in the formation of transport vesicles from the ER has been demonstrated [11–13]. The Saccharomyces Genome Database revealed that the hypothetical human protein encoded by KIAA0079 (KIAA0079p) [16] exhibits sequence similarity to yeast Sec24p. The two proteins share 25% identity over 878 amino acid residues. In the present study we tested whether KIAA0079p is a mammalian homologue of Sec24p. Biochemical and functional analyses revealed this to be the case. In addition, we showed that the C-terminal region of KIAA0079p is involved in the interaction with the N-terminal region of mammalian Sec23p.

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Abbreviations: COP, coat protein; ER, endoplasmic reticulum; GST, glutathione *S*-transferase; GFP, green fluorescent protein; mSec23p, mouse Sec23p; PAGE, polyacrylamide gel electrophoresis; VSVG, vesicular stomatitis virus-encoded glycoprotein

2. Materials and methods

2.1. Materials

Cell culture media and fetal bovine serum were obtained from Gibco BRL. An anti-FLAG antibody was obtained from Sigma. An anti-GST antibody, Superose 6, and glutathione-Sepharose 4B were obtained from Pharmacia Biotech. FITC-conjugated goat anti-rabbit IgG and rhodamine-conjugated goat anti-mouse IgG were purchased from Cappel Organon Teknika. Vero cells were from the Riken Cell Bank. Bovine brain cytosol was prepared as described previously [17]. A plasmid encoding KIAA0079p was obtained from the Kazusa DNA Research Institute, Japan.

2.2. Antibodies

A C-terminal region of KIAA0079p (amino acids 401–1125) was expressed in *Escherichia coli* as a His_6-tagged protein using a derivative of pQE30. The His_6-tagged protein expressed was almost exclusively localized in insoluble fractions. The insoluble proteins were isolated by centrifugation and separated by SDS-PAGE. The protein band corresponding to the recombinant protein was excised and injected into rabbits. The method for the preparation of anti-mSec23p will be described elsewhere.

2.3. Plasmids and transfection

Mammalian expression plasmids pEBG [18] and pFLAG-CMV-2 (Kodak) were used to express proteins fused with the N-terminal GST and N-terminal FLAG epitope, respectively. An expression plasmid for vesicular stomatitis virus-encoded glycoprotein-green fluorescent protein (VSVG-GFP) [19] was a kind gift from Dr. J. Lippincott-Schwartz (NIH). Transfection of plasmids was performed using LipofectAMINE PLUS according to the manufacturer's instructions.

2.4. In vitro binding assay

Expression plasmids were transfected into 293T cells [18]. At 24 h after transfection, the cells were lysed in lysis buffer (0.3 ml/35 mm dish) consisting of 25 mM HEPES-KOH (pH 7.0), 1% Triton X-100, 150 mM KCl, 0.5 μg/ml leupeptin, 2 μM pepstatin, 2 μg/ml aprotinin, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol. The lysates were clarified by centrifugation for 10 min at 15 000 rpm. The supernatant (250 μl) was incubated with glutathione-Sepharose 4B (15 μl) for 1.5 h at 4°C. The resultant beads were washed with the lysis buffer three times, and then the bound proteins were eluted with 20 μl of 2×SDS-PAGE sample buffer. The samples were separated by SDS-PAGE and immunostained with an anti-FLAG antibody using ECL (Amersham).

2.5. Immunofluorescence analysis

Immunofluorescence analysis was performed as described [20].

2.6. Transport of VSVG-GFP from the ER to the Golgi

The expression plasmid for VSVG-GFP was co-transfected with pFLAG-CMV-2 (control) or the plasmid for FLAG-KIAA0079p into Vero cells. The cells were incubated at 40°C for 24 h to allow the expression of proteins, and then shifted to 32°C for 30 min.

3. Results

3.1. KIAA0079p is associated with mammalian Sec23p

Immunoblotting analyses using an antibody against KIAA0079p showed that this protein is expressed in NRK

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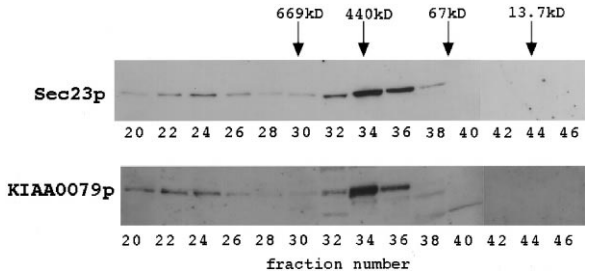

Fig. 1. Gel filtration of bovine brain cytosol on a Superose 6 column. Cytosol (2 mg) was loaded onto the column, and the fractions collected were subjected to SDS-PAGE, and analyzed by immunoblotting using antibodies against KIAA0079p and mSec23p. Vertical arrows indicate the elution positions of standards used to calibrate the column.

cells, 293T cells, and bovine brain (data not shown). The molecular mass of bovine brain KIAA0079p was estimated to be 120 kDa by SDS-PAGE analysis, which is consistent with its calculated value (121 kDa). The ubiquitous expression of KIAA0079p was confirmed by Northern blot analysis. The KIAA0079p probe detected a 4.4-kb transcript in human heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas (data not shown).

To examine whether KIAA0079p is associated with mammalian Sec23p, bovine brain cytosol was subjected to gel filtration on a Superose 6 column, and the eluates were analyzed with immunoblotting using antibodies against KIAA0079p and mSec23p. As shown in Fig. 1, the two proteins were co-eluted at a position corresponding to a molecular mass of approximately 300–400 kDa. This size is similar to that

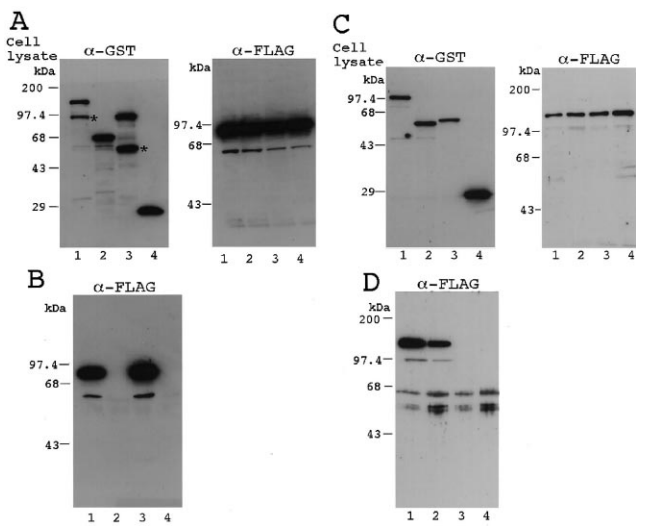

Fig. 2. In vitro binding analysis. A and B: The plasmid for GST-KIAA0079p (lane 1), GST-KIAA0079p-N (lane 2), GST-KIAA0079p-C (lane 3), or GST (lane 4) was cotransfected with the plasmid for FLAG-tagged mSec23p into 293T cells. To check the expression level, 4% of the lysate was analyzed with an anti-GST antibody (A, left panel) or an anti-FLAG antibody (A, right panel). Lysates of the transfected cells were incubated with glutathione beads, and the bound proteins were analyzed by immunoblotting with an anti-FLAG antibody (B). Asterisks in A represent proteolytic fragments of GST-KIAA0079p and GST-KIAA0079p-C. C and D: GST-mSec23p (lane 1), GST-mSec23p-N (lane 2), GST-mSec23p-C (lane3), or GST (lane 4) was coexpressed with FLAG-tagged KIAA0079p in 293T cells. Expression level and protein-protein interactions were analyzed as described above.

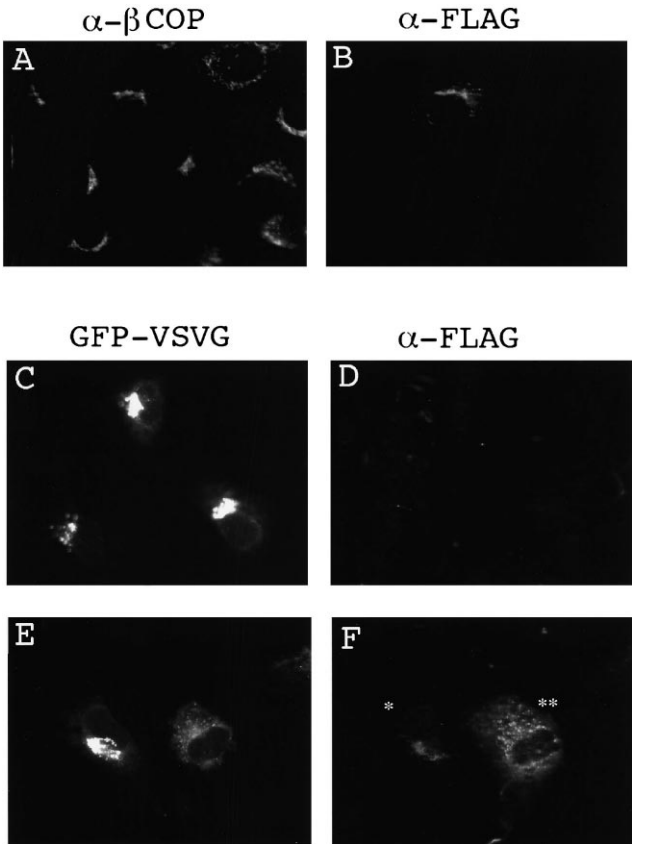

Fig. 3. Overexpression of KIAA0079p inhibits protein transport from the ER to the Golgi. Vero cells were transfected with the expression plasmid for FLAG-KIAA0079p. At 20 h after transfection, the cells were fixed and double-stained with anti-β-COP (A) and anti-FLAG (B) antibodies. To analyze the transport of VSVG-GFP from the ER, the plasmid for VSVG-GFP was transfected into Vero cells with the control plasmid pFLAG-CMV-2 (C and D) or the plasmid for FLAG-KIAA0079p (E and F). The cells were incubated at 40°C for 24 h, and then shifted to 32°C. After 30 min, they were fixed and stained with an anti-FLAG antibody followed by a rhodamine-conjugated secondary antibody. Fluorescence derived from GFP (C and E) and rhodamine fluorescence (D and F) is shown. Cells were double-labeled with GFP and rhodamine. Asterisk and double asterisk represent cells expressing low and high levels of FLAG-tagged KIAA0079p, respectively. KIAA0079p when expressed at higher levels was localized throughout the cells.

of the yeast Sec23p-Sec24p complex [5]. Minor amounts of the two proteins that eluted at a position corresponding to a higher molecular mass may represent protein aggregates.

3.2. Interaction between KIAA0079p and mSec23p

To confirm the association of KIAA0079p with mammalian Sec23p, and also localize regions involved in the association, GST-KIAA0079p (full-length), GST-KIAA0079p-N (amino acids 1–400), or GST-KIAA0079p-C (amino acids 401–1125) was coexpressed with FLAG-tagged mSec23p in 293T cells (Fig. 2A). Cell lysates of each transfectant were incubated with glutathione beads, and the proteins bound to the beads were analyzed by immunoblotting using an anti-FLAG antibody. As shown in Fig. 2B, FLAG-tagged mSec23p bound to GST-KIAA0079p (lane 1) and GST-KIAA0079p-C (lane 3), but not GST-KIAA0079p-N (lane 2). The interaction between KIAA0079p and mSec23p was tight, and the complex did not dissociate upon 1 M NaCl treatment (data not shown). A

yeast two-hybrid assay revealed that their interaction is direct (data not shown).

Similar experiments were performed to define the region of mSec23p involved in the interaction with KIAA0079p. GST-mSec23p (full-length), GST-mSec23p-N (amino acids 1–359), or GST-mSec23p-C (amino acids 360–765) was coexpressed with FLAG-tagged KIAA0079p, and interaction of the expressed proteins was analyzed. As shown in Fig. 2D, the N-terminal region of mSec23p is responsible for the association with KIAA0079p.

3.3. Implication of KIAA0079p in protein export from the ER

We next examined the subcellular localization of KIAA0079p. FLAG-tagged KIAA0079 was transiently expressed in Vero cells, and its distribution was analyzed with immunofluorescence microscopy. When expressed at low levels, FLAG-tagged KIAA0079p was located in structures scattered throughout the cytoplasm and in more central reticular structures (Fig. 3B), significantly colocalizing with a *cis*-Golgi marker protein, β-COP (Fig. 3A) [21,22]. This distribution seemed to coincide with those of proteins in the ER-Golgi intermediate compartment [23,24], consistent with the idea that KIAA0079p is involved in protein export from the ER.

To provide evidence for the involvement of KIAA0079p in protein transport from the ER to the Golgi apparatus, we examined the effect of overexpression of KIAA0079p on the transport of the ts045 VSVG tagged with GFP (VSVG-GFP). A previous study showed that VSVG-GFP expressed in cells is exclusively retained in the ER at non-permissive temperature (40°C), and is transported from the ER to the plasma membrane via the Golgi apparatus upon lowering the temperature to 32°C [19]. The plasmids for VSVG-GFP and FLAG-tagged KIAA0079p were transfected into Vero cells, and the cells were incubated at 40°C for 24 h. In a control experiment pFLAG-CMV-2 was transfected instead of the plasmid for FLAG-tagged KIAA0079p. Expressed VSVG-GFP was exclusively localized in the ER at 40°C regardless of the presence of KIAA0079p (data not shown). Upon temperature shift to 32°C for 30 min, VSVG-GFP was transported to the Golgi apparatus in control cells (Fig. 3C) or in cells expressing lower levels of KIAA0079p (Fig. 3E), whereas it was not exported from the ER in cells expressing higher levels of KIAA0079p (Fig. 3E).

4. Discussion

Several lines of evidence suggest that KIAA0079p interacts with mammalian Sec23p. First, the KIAA0079p was co-eluted with mammalian Sec23p on Superose 6 gel filtration. Second, the direct interaction between the two proteins was observed in a yeast two-hybrid assay. Third, FLAG-tagged mSec23p was coprecipitated with GST-KIAA0079p, and FLAG-tagged KIAA0079p, in reverse, was coprecipitated with GST-mSec23p.

The N-terminal region of KIAA0079p comprising about 330 amino acids is rich in proline (22%). Proline-rich regions are often involved in protein-protein interactions [25]. Indeed, we have recently identified a mammalian Sec23p-interacting protein, p125, and found that its proline-rich region is involved in the interaction with mammalian Sec23p (unpublished results). However, the N-terminal proline-rich region of KIAA0079p did not bind to mSec23p. Given that the mSec23p-KIAA0079p complex did not dissociate upon 1 M NaCl treatment, the interaction between the two proteins is not ionic, but may be hydrophobic. However, further studies are required to address this issue.

Overexpression of KIAA0079p blocked the export of VSVG-GFP from the ER. There are several possibilities to explain this inhibitory effect. One possible explanation is that excess KIAA0079p hinders the interaction between the Sec23p-Sec24p complex and other protein(s) involved in vesicle formation. The most likely candidate is Sec16p. Yeast Sec16p is a peripheral ER membrane protein and is essential for the formation of COPII-coated vesicles [26]. It may be a component of COPII-coated vesicles [27] or may remain on the ER membrane for organizing budding sites [28]. Sec23p and Sec24p can interact independently with Sec16p to form a ternary complex in vitro [29]. Free KIAA0079p may compete with the Sec23p-KIAA0079p complex for the binding site on Sec16p, leading to the inhibition of vesicle formation. Another possibility is that the overexpression of KIAA0079p inhibits individual functions of multi-species of mammalian Sec24p. Given the presence of three closely related Sec24 proteins in yeast, multi-species of Sec24p may be present in mammalian cells. It is possible that the function of each species is important for vesicle formation, and overexpression of one species may disturb the functions of other species. Although the exact molecular explanation for the inhibitory effect of the overexpression of KIAA0079p is not clear at present, our results suggest the involvement of KIAA0079p in protein export from the ER.

Taken together the present results strongly suggest that KIAA0079p is a mammalian homologue of Sec24p. During the course of this study Aridor et al. [30] reported the presence of mammalian Sec24p in a complex containing mSec23p in rat liver cytosol. This protein may be the same as KIAA0079p.

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