p125 Is a Novel Mammalian Sec23p-interacting Protein with Structural Similarity to Phospholipid-modifying Proteins

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The transport of proteins between intracellular compartments is mediated by vesicles that bud from the donor compartment and move to the target compartment, where the fusion of vesicles occurs (1–3). COPII-coated vesicles mediate protein transport from the ER to the Golgi apparatus. Genetic and biochemical analyses of Saccharomyces cerevisiae showed that seven proteins, Sar1p, Sec12p, Sec16p, Sec13p, Sec23p, Sec31p, and Sec24p, are involved in the formation of vesicles from the ER (4–9). It has been reported that Sar1p, the Sec13p-Sec31 complex, and the Sec23p-Sec24p complex are components of COPII (10). Sar1p is a small GTP-binding protein and exists in the cytosol in the GDP-bound form. Sec12p, which exists in the ER membrane (10), exchanges GDP in Sar1p with GTP. The resultant Sar1p-GTP binds to the ER membrane, and then sequentially recruits Sec23p-24p and Sec13p-31p to the membrane. The recruitment of coat proteins leads to vesicle budding from the ER membrane (11, 12). Once vesicles are formed, Sec33p acts as a GTPase-activating protein (GAP) for the Sar1p GTPase under the uncoating process (13). Thus, the cycle of Sar1p GTP hydrolysis regulates the formation and uncoating of COPII-coated vesicles (14). The role of Sec16p in the vesicle formation is not clear. Like the Sec23p-24p and Sec13p-31p complexes, Sec16p may be a component of the vesicle coat (15–17). Alternatively, it may organize budding sites and remain on the ER membrane even after vesicle formation (18).

Mammalian homologues of Sar1p (19), Sec13p (20, 21), and Sec23p (22, 23) were identified, and their involvement in the formation of transport vesicles from the ER membrane has been demonstrated (19–21, 24). We are especially interested in mammalian Sec23p because it has unique features. Sec23p mediates vesicle formation as a component of coat proteins, and thereafter acts as a GAP for Sar1p to promote uncoating. In this study we tried to identify new mouse Sec23p-interacting proteins using an affinity isolation method. We found a novel mammalian Sec23p-interacting protein, p125, which shows significant homology with phospholipid-modifying proteins, especially phosphatidic acid-prefering phospholipase A1.

EXPERIMENTAL PROCEDURES
cDNA Cloning and Sequencing of Mouse Sec23p—Wadhwa et al. (25) reported that a mouse cDNA clone, Msec23, encodes a protein that exhibits similarity to yeast Sec23p. The calculated molecular mass of the Msec23 protein is smaller than that of yeast Sec23p. We re-examined the DNA sequence of the Msec23 clone and found a sequencing error. This causes a frameshift leading to the early termination codon. The full-length cDNA was obtained by screening a mouse brain AZAPII cDNA library (Stratagene Inc.) with the Msec23 clone as a probe. The full-length cDNA encodes a protein that is 99% identical to human Sec23Ap (23), and hence this protein is referred to as mouse Sec23p.

Expression and Purification of the GST-Sec23p Fusion Protein—Mouse Sec23p was expressed as a GST fusion protein in Sf9 cells with the baculovirus expression system. The coding region of mouse Sec23p was inserted into the pAcG2T plasmid (PharMingen) using the EcoRI-BamHI site in frame with the upstream GST sequence, and the resultant plasmid was used as a transfer vector. Recombinant viruses were obtained using Linearized BaculoGold DNA (PharMingen) according to the manufacturer’s instructions. For the expression of a GST protein, pAcG2T was used as a transfer vector and recombinant viruses were obtained in the same way.

Sf9 cells were maintained in SF-900II SFM basal medium (Life Technologies, Inc.) supplemented with gentamicin sulfate (50 μg/ml) at 25 °C. Cells were infected with recombinant virus at a multiplicity of infection of 10. After 48 h, the cells were harvested by centrifugation, and then lysed in lysis buffer comprising 20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1% Nonidet P-40 (Calbiochem), 1 mM PMSF, 1 μg/ml leupeptin, 2 μM pepstatin, 2 μg/ml aprotinin, 10 μg/ml trypsin inhibitor, and 1 mM EDTA. The lysates were clarified by centrifugation at 38,000 rpm (Beckman 70.Ti rotor) for 30 min, and then incubated with glutathione-Sepharose 4B (Amersham Pharmacia Biotech) at 4 °C for 20 min. The
beads were washed three times with the lysis buffer, and then two buffers with 50 mM Tris-HCl (pH 8.0) and 0.1% Triton X-100. The proteins were eluted with 0.1% Triton X-100 and 250 mM glutathione (the pH was adjusted to neutral with Tris base). The eluent was collected, and then dialyzed against 50 mM Tris-HCl (pH 8.0) and 0.1% Triton X-100. The solution was pre-cleared, and then homogenized three times with a Potter-Elvehjem homogenizer and then precipitated with 10% trichloroacetic acid (pH 7.5) and 320 mM sucrose, and then homogenized three times with a Potter-Elvehjem homogenizer in three volumes of 25 mM Tris-HCl (pH 8), 250 mM KCl, 250 mM sucrose, 1 mM DTT, 2 mM EDTA, 0.5 μg/ml leupeptin, 2 μM peptatin A, 1 mM PMSF, 2 μM aprotinin, and 0.5 mM 1,10-phenanthroline. Triton X-100 was added to a final concentration of 2%, and then the homogenate was kept on ice for 1 h. The homogenate was centrifuged at 38,000 rpm (Beckman 70.Ti rotor) for 30 min, and the supernatant was incubated with glutathione-Sepharose 4B at 4 °C for 2 h to remove proteins that bind to this resin. The resin was removed by centrifugation, and the supernatant was used as the mouse brain lyseate.

Affinity Isolation of Mouse Sec23p-interacting Proteins—In a typical experiment, 50 μl of glutathione-Sepharose 4B (50 μg) or GST protein (5 μg) or protein GST-Sec23p (about 4 μg) or GST protein at 4 °C for 20 min. The beads were washed twice with 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.1% Triton X-100, and then twice with washing buffer comprising 25 mM Hepes-KOH (pH 7.0), 150 mM KCl, 0.75% Triton X-100, 1 mM DTT, 2 mM EDTA, and 0.5 mM PMSF. The eluate was collected, and then the eluent was centrifuged at 15,000 rpm for 30 min. The supernatant (about 4 μg of protein) was mixed with glutathione-Sepharose 4B that had bound GST-Sec23p or GST, followed by incubation at 4 °C for 3 h. The beads were then washed four times with the washing buffer. The proteins were eluted with 0.1% Triton X-100 and 250 mM glutathione (the pH was adjusted to neutral with Tris base), and then precipitated with 10% trichloroacetic acid. The precipitate was washed with acetone and then analyzed by SDS-PAGE.

cDNA Cloning and Sequencing of p125—Polypeptides separated by SDS-PAGE were electroblotted onto a ProBlotTM membrane (Applied Biosystems) and then stained with 2% Ponceau S in 1% acetic acid. The protein band corresponding to an apparent molecular mass of 125 kDa on the membrane was excised and sequenced as described previously (27). The EST data base was searched with the BLAST program using the peptide sequences derived from p125. The search revealed a human EST clone (accession no. T05457) that encodes a protein containing one identified peptide sequence. This clone was obtained from the ATCC EST clone (accession no. T05457) that encodes a protein containing one stretch plus cDNA library (CLONTECH). Three strong positive clones were obtained and that with the longest cDNA insert (4.3 kb) was sequenced. The 5′-RACE method was performed using a Marathon™ cDNA amplification kit (CLONTENGE) according to the manufacturer's instructions. Marathon-Ready™ cDNA from human placenta and a synthetic oligonucleotide (5′-TTTTCCTTTTGGCGCCCGCCTGCAACTTGAGGCACTCCTCCCGGCTGG) were used for PCR as a template and an antisense primer, respectively. The primer was complementary to nucleotides encoding amino acid residues 436–444.

Antibodies—A peptide corresponding to amino acid residues 348–361 (KGDTDSRFIPYTEE) of p125 was synthesized with an additional lysine residue to keyhole limpet hemocyanin and used to immunize rabbits. An anti-p125 antibody was affinity-purified from the sera with peptide-coupled Sepharose 4B.

Mouse Sec23p with a C-terminal histidine tag was expressed in Escherichia coli using the expression vector pET3a-derivative, which was prepared by Sangaard et al. (28). The N-terminus of mouse Sec23p was changed from Met-Thr-Thr to Met-Gly-Ser-Thr-Thr, and its C terminus contained a Gly-Ser-His6 tag. The protein was expressed in BL21 (DE3) E. coli cells (Novagen) and purified from inclusion bodies on a Ni-NTA column. Rabbits were immunized with the purified protein, and the antibody was affinity-purified using an antigen-Sepharose 4B column.

The monoclonal anti-ERGIC-53 antibody was a generous gift from Dr. H.-P. Hauri of the University of Basel. The polyclonal anti-yeast Sec18p antibody was a generous gift from Dr. C. A. Kaiser of the Massachusetts Institute of Technology. The polyclonal anti-β-COP was donated by T. Yamaguchi of this laboratory. The anti-FLAG antibody and anti-GST antibody were obtained from Eastman Kodak Corp. and Amersham Pharmacia Biotech, respectively.

Northern Blot Analysis—A human multiple-tissue blot of poly(A)+ RNA was purchased from CLONTECH. The cDNA fragment encoding the N-terminal fragment (residues 1–367) of p125 was amplified by PCR and used as a probe. For analysis of mammalian Sec23p, the cDNA fragment encoding residues 69–252 of mouse Sec23p was amplified by PCR and used as a probe. Hybridization was carried out overnight at 65 °C in 5× SSPE according to the manufacturer's instructions. The blot was washed for 40 min in 2× SSC with 0.05% SDS at room temperature, and then for 40 min in 0.1× SSC with 0.1% SDS at 50 °C. Radioactivity was detected with a Fuji Bioimage analyzer BAS2000.

Cell Culture—Vero cells or 293T cells (29) were maintained in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) supplemented with 50 IU/ml penicillin, 50 μg/ml streptomycin, and 10% fetal calf serum.

Plasmid Construction and Transfection—Mammalian expression plasmid pEG-βgal (pho-disincubating 29) and pFLAG-CMV-2 (Eastman Kodak Corp.) were used to express proteins fused with the N-terminal GST and N-terminal FLAG epitope, respectively. The cDNA fragment encoding the full-length mouse Sec23p was inserted into pEGB. The cDNA fragment encoding the full-length p125 (residues 1–1000), N-terminal fragment (residues 1–367), or C-terminal fragment (residues 368–1000) was inserted into pFLAG-CMV-2.

For the expression of fusion proteins, 293T cells plated on 35-mm dishes were transfected with 1–2 μg of expression plasmids using the LipofectAMINE reagent (Life Technologies, Inc.) according to the manufacturer's instructions. At 24 h after transfection, the cells were lysed in lysis buffer (0.35 ml/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 PMSF, and 1 mM DTT. The lysates were clarified by centrifugation for 10 min at 15,000 rpm and then used for the binding assay.

In Vitro Binding Assay—In a typical experiment, 250 μl of a cell lysate was incubated with 15 μl of glutathione-Sepharose 4B for 1.5 h at 4 °C. The resultant beads were washed with the lysis buffer three times, and then the proteins were eluted with 20 μl of 2× SDS-PAGE sample buffer. The samples were separated by SDS-PAGE and immunostained with an anti-p125, anti-GST, or anti-FLAG antibody using ECL (Amersham Pharmacia Biotech).

Two-hybrid Analysis—The MATCHMAKER™ two-hybrid system (CLONTECH) was used. The full-length coding region of mouse Sec23 was subcloned into yeast expression vector pGAL9, and the full-length (residues 1–1000) or N-terminal (residues 1–367) coding region of p125 was subcloned into pGAD424. Yeast strain SP7526 was transformed with bait and prey vectors using the lithium acetate method, and then plated on selection plates lacking tryptophan and leucine. Filter assays for β-galactosidase activity were performed according to the manufacturer’s instructions.

Immunofluorescence Microscopy—Immunofluorescence microscopy was performed as described previously (30). Briefly, cells plated on coverslips were fixed with 4% paraformaldehyde, followed by sequential incubation with the primary antibodies and FITC-conjugated or rhodamine-conjugated secondary antibody. For staining using the anti-Sec23p or anti-p125 antibody, 2% paraformaldehyde followed by methanol was used for fixation. For staining of the Golgi apparatus in Vero cells, FITC-conjugated Lentinus culinaris lectin (E-Y Laboratories) was used.
Novel Sec23p-interacting Protein

MAERPKFNGSSGASTSSSGLRTWLLFSFHAVPFVITVQAASSASPASLA 50
LLFGEDSTVGEEEDSPLLGLQTIIATTSQGTSFYQVSQVSSDPFGIQQGSP 100
LTAYTVGSGGCFLKPLTALPTTGGQGVSNASSFKQAKGAPPSLSSM 150
GINSYLPSPQSLPLSFYQGFPGQFQGQVNYRVQTSRGPSSRANYPAPQ 200
LQCCOTPGPAHIPPPFSGPVPQGMSLPPVQSVQVQQVFPAPRRKFG 250
AFCSPYSFSPFLQQEYFQHFCYVEQKVLQWSVTSVSLMEETY 300
NSQAPSP5SGSILGQDGRYKFLVYDRIOSAMYWDEAFTRCRVFWYGRQ 350
TDSRFFTFYTEERSEKKAEYKAVANTQEQWIRPLFESGTVQMVPPFQVUV 400
QOFPSVSEWBG7QDQTQPRVRGKDIDNIDE1DPGMQPFVHILFVY 450
HIGFCVDCLIMFRSLIICVDFDRFV813LLES7RKPSLDQQKVSKVFNF1PV 500
EHMSLGLGADTVDGRNkirTLPSGFRSBFETN2LDLFGYNSPCYTQCT 550
IVEXVGMEMHLHALFMSNRPDKGQGVXAVXHSHLDQFLDPS15MKCDN 600
LSKCPGFLAVANGVYQKLFQEPFEEKITLEIDESVLYVEKVTILQ 650
ETLARSLISYEYTFSEKEDMDSLMLCTVDVLLKKGPIGFLDQKiantsV 700
KHHAKAIKLXANKXAVEATSTQGQCAEKTQDNLSPSSEDEPKEKIP 750
VGACVYSSCYNFRSFEQVAQGVSAYNSDLFEPEFIEALGFSPNMTFLIR 800
GVRCRINTQLSLETCKOFYTHLDPFAYKELERPIMVPDLKAVLILPHK 850
GRKELRLIESLDSQGSDLACGFISASLAMQTNABAKARHTSSTQAC 900
ELKCVANGKLKEEKSEYAEVAYKEDPSKDEYKKVQMLHSGIRYDK 950
VLQKKFIESTYFIALGFSLCHLYWESCTALLKLGKYN11FESPOCR 1000

Ser-Leu-Gly565, match the consensus sequence present in most lipases, Gly-X-Ser-X-Gly, where X represents any amino acid (32). Third, residues 883–925 near the C terminus are predicted to form a coiled-coil region with a score of 1.00 according to the Lupas algorithm with the 28-residue window (33).

Sequence Homology between p125 and Phospholipase A₁—
The non-redundant protein data base was searched with the BLAST program using the amino acid sequence of p125 as a probe. The results revealed that the central and C-terminal regions of p125 encompassing about 700 amino acid residues exhibit sequence similarity with several proteins, including phosphatidic acid-prefering phospholipase A₁ (34), and three unknown proteins from Caenorhabditis elegans (GenPept: CELM03A_5), Schizosaccharomyces pombe (gp: SPACC0G8_2), and S. cerevisiae (YOR022c). The latter three proteins were identified during genome sequencing of the respective organisms. Recently, it was suggested that these four proteins are members of a new lipase family (34). Fig. 3A shows the sequence alignment of p125 and phosphatidic acid-prefering phospholipase A₁. Fig. 3B shows the sequence alignment of p125, phosphatidic acid-prefering phospholipase A₁, and the three unknown proteins from C. elegans, S. pombe, and S. cerevisiae.

The data base search also revealed weak homology between p125 and retinal degeneration B protein (RdgBp). RdgBp was originally identified as a protein of which a mutation causes retinal degeneration in Drosophila (35). This protein contains a region homologous to phosphatidylinositol transfer protein at the N terminus and actually exhibits such transfer activity (for a review, see Ref. 36).

Northern Blot Analysis of p125—We examined the tissue expression pattern of p125 by Northern blot analysis. As shown in Fig. 4A, the p125 probe revealed a 4.5-kb transcript in all tissues examined, indicating that p125 is expressed ubiquitously. The expression of p125 is relatively low in brain, lung, and kidney. This expression pattern was similar to that of Sec23p (Fig. 4B). In addition, a 1.8-kb transcript of p125 was also observed in tissues expressing higher amounts of the 4.5-kb transcript (Fig. 4A). Interestingly, a similar smaller transcript was also observed among the transcripts of Sec23p (Fig. 4B). At
this moment, it is not clear whether these smaller transcripts are degradation products of the larger transcripts or alternative splicing products of Sec23p and p125.

The N-terminal Region of p125 Is a Major Site Involved in the Association with Sec23p—We wanted to confirm the interaction between Sec23p and p125 by immunoprecipitation. Unfortunately, antibodies prepared against the two proteins worked for Western blotting but not for immunoprecipitation. We therefore transiently expressed GST-Sec23p in cultured cells and examined its interaction with endogenous p125. GST or GST-Sec23p was expressed in 293T cells (Fig. 5A, bottom panel), and cell lysates were prepared and incubated with glutathione beads. The proteins bound to the beads were analyzed by immunoblotting using an anti-p125 antibody. As shown in Fig. 5A (top panel), endogenous p125 bound to the beads when the lysates were prepared from the GST-Sec23p-expressing cells, but not from the GST-expressing cells. The amount of p125 bound to the GST-Sec23p beads was about 5% of the total p125 existing in the cell lysates. This result may suggest that p125 is a minor Sec23p-interacting protein. When FLAG-tagged Sec23p was expressed and precipitated with an anti-FIG. 4. Northern blot analysis. A poly(A)⁺ mRNA human multiple tissue blot was probed with ³²P-labeled cDNA of p125 (A) or Sec23p (B).
Novel Sec23p-interacting Protein

Fig. 5. The N-terminal region of p125 is involved in the interaction with Sec23p. A, the expression plasmid for GST (lane 1) or GST-Sec23p (lane 2) was transfected into 293T cells. Lysates of the transfected cells were incubated with glutathione beads, and the bound proteins were analyzed by SDS-PAGE and immunoblotting with an anti-GST antibody. One percent of the lysates of the GST-Sec23p expressing cells was blotted on the first lane from the left. The position of p125 is indicated (top). GST and GST-Sec23p in the lysates were detected with an anti-GST antibody. The positions of GST-Sec23p and GST are indicated (bottom). B, the expression plasmid for GST-Sec23p (lanes 1, 2, and 3) or GST (lanes 4, 5, and 6) in combination with the expression plasmid for FLAG-p125 (residues 1–1000) (lanes 1 and 4), FLAG-p125-N (residues 1–367) (lanes 2 and 4), or FLAG-p125-C (residues 368–1000) (lanes 3 and 6) were cotransfected into 293T cells. One μg of the plasmid for GST or GST-Sec23p was used. To adjust the expression levels of FLAG-tagged proteins, 0.025 μg of the plasmid for FLAG-p125 or FLAG-p125-N was used with 0.975 μg of FLAG-CMV-2 (lanes 1, 2, 4, and 5). One μg of the plasmid for FLAG-p125-C was used (lanes 3 and 6). Lysates of the transfected cells were incubated with glutathione beads, and the bound proteins were analyzed by immunoblotting with an anti-FLAG antibody (top). Four percent of the lysates was analyzed by immunoblotting with the anti-FLAG antibody (bottom). The positions of FLAG-p125, FLAG-p125-N, and FLAG-p125-C are indicated.

 FLAG antibody, endogenous p125 was coprecipitated (data not shown).

To define the region of p125 involved in the interaction with Sec23p, GST-Sec23p and FLAG-tagged p125 or its derivatives were coexpressed in 293T cells (Fig. 5B). Each of the plasmids encoding the full-length p125 (FLAG-p125: residues 1–1000), the N-terminal domain (FLAG-p125-N: residues 1–367), and the C-terminal domain (FLAG-p125-C: 368–1000) was cotransfected with a plasmid encoding GST-Sec23p. Cell lysates of each transfected cell were incubated with glutathione beads, and the bound proteins were analyzed by immunoblotting using the anti-FLAG antibody. As shown in Fig. 5B (top panel), FLAG-p125 and FLAG-p125-N bound to GST-Sec23p. Binding of FLAG-p125-C to GST-Sec23p was marginally detected. These results suggest that the N-terminal region is a major site involved in the interaction with Sec23p. When FLAG-p125 or FLAG-p125-N was coexpressed with GST, neither protein bound to the beads, confirming the specific interaction of FLAG-p125 and FLAG-p125-N with GST-Sec23p.

In order to confirm that p125 interacts directly with Sec23p, we used the yeast two-hybrid system (Fig. 6). Mouse Sec23p was fused to the GAL4 DNA-binding domain, and the full-length (residues 1–1000) or N-terminal domain (residues 1–367) of p125 was fused to the GAL4 activation domain. These two types of constructs were cotransfected into yeast SPY526, and then the interaction was examined by assaying the activation of transcription of a lacZ reporter gene. β-Galactosidase activity was induced when pGAD424-p125 or pGAD424-p125N was cotransformed with pGBT9-Sec23p. In contrast, no induction was observed when pGAD424-p125 or pGAD424-p125N was cotransformed with pGBT9. In addition, there was no induction of β-galactosidase activity when pGBT9-Sec23 was cotransformed with pGAD424. These results suggest that the N-terminal region of p125 interacts directly with Sec23p.

Subcellular Distribution of p125—In order to determine the subcellular distribution of p125, we first performed subcellular fractionation. Homogenates of rat brains or NRK cells were centrifuged at 1,000 × g for 5 min, yielding post-nuclear supernatant and nuclear fractions. The post-nuclear supernatant was then centrifuged at 9,000 × g for 10 min, yielding supernatant and mitochondrial fractions. The supernatant was further centrifuged at 105,000 × g for 1 h, yielding microsomal and cytosolic fractions. The proteins in each fraction were resolved by SDS-PAGE, and then immunoblotted with anti-p125 (Fig. 7). The results showed that p125 is localized predominantly in the microsomal and cytosolic fractions. p125 appeared to be a very labile protein. Several degradation products were observed even when protease inhibitors were included during the homogenization and subcellular fractionation. Two bands corresponding to molecular weights lower than that of p125 were observed for the mitochondrial fraction of NRK cells. They might be degradation products of p125 or other proteins, but we did not investigate them further.

Overexpression of p125 Causes Dispersion of the Golgi Apparatus—Next, we tried to localize p125 by immunofluorescence analysis. FLAG-tagged p125 was transiently expressed in Vero cells, and then its subcellular distribution was analyzed by immunofluorescence microscopy. Because endogenous staining with an anti-p125 antibody was weak, transient expression was necessary. The localization of p125 was dependent on the level of expression of p125. P125 was colocalized with β-COP (Fig. 8A and B), a subunit of COP1 (37, 38), and ERGIC-53 (Fig. 8E and F), a marker protein for the ER-Golgi intermediate compartment (39, 40), in cells expressing lower levels of p125. In cells expressing higher amounts of p125, the protein was detected throughout cells, reflecting its localization not only in membranes but also in cytosol (Fig. 8C, G, and I). In these cells, β-COP (Fig. 8D) and ERGIC-53 (Fig. 8H) exhibited dispersed staining patterns. The Golgi apparatus (Fig. 8J), which was visualized with FITC-conjugated L. culinaris lectin, was also dispersed. Perturbation of the ER-Golgi intermediate compartment and Golgi apparatus is not peculiar to the overexpression of p125. When Sec23p was overexpressed, similar diffusive patterns were observed for β-COP (Fig. 9A and B), ERGIC-53 (Fig. 9C and D), and the Golgi apparatus (Fig. 9E and F). Similar results were obtained when baby hamster kidney cells were used (data not shown). These observations suggest that p125 is involved in the early secretory pathway.
In this study we identified a novel mouse Sec23p-interacting protein, p125, and determined its cDNA sequence. Several lines of evidence suggest that p125 interacts specifically with mammalian Sec23p. First, p125 in mouse brain lysates bound to GST-Sec23p-beads. Second, p125 was coprecipitated with GST-Sec23p and FLAG-tagged Sec23p expressed transiently in cultured cells. Third, the direct interaction between p125 and Sec23p was demonstrated by means of the yeast two-hybrid assay. Furthermore, we found that the N-terminal 367-amino acid region of p125, which is rich in proline residues, is a major site for the interaction with Sec23p.

It was reported that yeast Sec23p binds to Sec24p (5, 41). These proteins form a 300–400-kDa protein complex, which promotes vesicle formation (5). Recently, Balch and colleagues reported the isolation of the Sec23p-Sec24p complex from rat liver cytosol (24). Rat Sec24p is a 120-kDa protein, which is close in size to p125. In our experiments, however, Sec24p was not coprecipitated with GST-Sec23p from mouse brain lysates. It is not clear why we did not detect the mammalian Sec24p in our experiments. One possibility is that Sec24p is poorly expressed in mouse brain or very labile in homogenates. The eluent from GST-Sec23p beads might contain only a small amount of Sec24p. It is known that both yeast and mammalian Sec24p are susceptible to proteolysis (24, 41). Another possibility is that all Sec24p expressed in mouse brain is bound tightly to Sec23p. Therefore, Sec24p in the complex may not bind to the free GST-Sec23p.

The Saccharomyces Genome Database revealed that the human cDNA clone, KIAA0079 (42), which encodes a 121-kDa protein, exhibits sequence similarity to that of yeast Sec24p. Since yeast Sec24p and KIAA0079 exhibit high sequence homology ($P_N = 7.5 \times 10^{-42}$ on a BLAST search) and have similar molecular weights, the KIAA0079 protein could be a mammalian Sec24p. To test this possibility, we prepared an expression plasmid for the KIAA0079 protein and examined the interaction of this protein with mouse Sec23p in cultured cells. Preliminary results suggest that the KIAA0079 protein is a major

**Fig. 7.** p125 is localized in the membrane and cytosol fractions. Homogenates of rat brains or NRK cells were subjected to subcellular fractionation. Proteins (50 μg) in each fraction were analyzed by SDS-PAGE and immunoblotting with an anti-p125 antibody.

**Fig. 8.** Overexpression of p125 causes disorganization of the ER-Golgi intermediate compartment and Golgi apparatus. Vero cells were transfected with the expression plasmid for FLAG-tagged p125. At 24 h after transfection, the cells were fixed and double-stained with anti-FLAG (A and C) and anti-β-COP (B and D), anti-p125 (E and G) and anti-ERGIC-53 (F and H), or anti-FLAG (I) and L. culinaris lectin (J).

**Fig. 9.** Expression of Sec23p also causes disorganization of the ER-Golgi intermediate compartment and Golgi apparatus. Vero cells were transfected with the expression plasmid for FLAG-tagged Sec23p. At 24 h after transfection, the cells were fixed and double-stained with anti-FLAG (A) and anti-β-COP (B), anti-sec23p (C) and anti-ERGIC-53 (D), or anti-FLAG (E) and L. culinaris lectin (F).
Sec23p-interacting protein and more tightly associated with Sec23p than p125 (58). However, additional studies are needed to clarify the interaction of mammalian Sec23p with the KIAA0079 protein and p125.

Overexpression of p125 in cultured cells altered the localization of ERGIC-53, a marker protein for the ER-Golgi intermediate compartment (39, 40) and β-COP, a cis-Golgi protein (37, 43). In addition, the Golgi apparatus was dispersed in the cells overexpressing p125. These findings suggest that p125 might play a role in the maintenance of the ER-Golgi intermediate compartment or Golgi structures. Another explanation for this phenomenon is that overexpression of p125 perturbs the membrane traffic between the ER and Golgi apparatus, which results in dispersion of the Golgi apparatus in an indirect way. When the formation of vesicles from the ER membrane is blocked by the expression of a mammalian Sar1p mutant that preferably binds GDP, the localization of ERGIC-53 is altered (19, 44). Similarly, β-COP and the Golgi apparatus become dispersed upon the expression of an inactive ARF1 mutant (45). ARF1 is a small GTP-binding protein required for the formation of COPI-coated vesicles (46). On the other hand, expression of an active mutant of Sar1p (19, 44) or ARF1 (44, 47) does not cause Golgi disassembly. The phenotype of cells overexpressing p125 is quite similar to that of cells expressing an inactive form of Sar1p or ARF1, suggesting that overexpression of p125 inhibits vesicle formation. Consistent with this idea, overexpression of Sec23p, which is a GAP for Sar1p, yielded a similar phenotype to that observed for cells overexpressing p125. It should be noted that this morphological change does not always occur when proteins existing between the ER and Golgi apparatus are overexpressed. No alteration occurs in the ER-Golgi intermediate compartment or Golgi apparatus when ERGIC-53 (48) or its mislocalized mutant (49) is overexpressed.

p125 has a protein motif for lipases. A data base search revealed that it exhibits sequence homology with phospholipid-modifying proteins, especially phosphatidic acid-prefering phospholipase A1 from bovine testis (34). This lipase, also containing a coiled-coil region, is definitely unique among lipases. It preferentially catalyzes the hydrolysis of phosphatidic acid in mixed micelle assay systems (50), and no significant homology was found with types I–IV phospholipase A2, a phosphatidic acid-preferring protein, which participates in the early secretory pathway. Measurement of the enzyme activity of p125 is currently under way to examine this intriguing possibility.

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