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Determination of Functional Regions of p125, a Novel Mammalian Sec23p-Interacting Protein

Toshihide Mizoguchi,* Ken-ichi Nakajima,* Kiyotaka Hatsuzawa,* Masami Nagahama,* Hans-Peter Hauri,† Mitsuo Tagaya,* and Katsuko Tani*,[1]

**School of Life Science, Tokyo University of Pharmacy and Life Science, Hachioji, Tokyo 192-0392, Japan; and*
†Department of Pharmacology, Biozentrum, University of Basel, CH-4056 Basel, Switzerland

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The Sec23p–Sec24p complex is a component of coat protein II-coated vesicles involved in protein export from the endoplasmic reticulum. We previously identified a novel Sec23p-interacting protein, p125, which consists of 1000 amino acids and comprises a proline-rich region and a phospholipase A_1 homology region. p125, when ectopically expressed in cultured cells, localizes to endoplasmic reticulum-Golgi intermediate regions. In the present study we showed that expressed p125 principally colocalizes with p115 and GM130, both of which are involved in vesicle tethering to Golgi membranes. Next, we determined the functional regions of p125 by expressing a p125 series with deletions. The results showed that the proline-rich region (residues 135–259) is responsible for the binding to Sec23p. For the correct localization of p125, a region (residues 135–1000) comprising both the proline-rich and phospholipase A_1 homology regions was required.

Key Words: **endoplasmic reticulum; Golgi apparatus; proline-rich region; phospholipase A_1; Sec23p.**

Protein transport between the endoplasmic reticulum (ER) and Golgi is mediated by two types of transport vesicles, coat protein I (COPI)- and coat protein II (COPII)-coated vesicles (1, 2). COPII vesicles are involved in the export of secretory and membrane proteins from the ER. In mammalian cells, the COPII seems to be replaced with COPI in the transit to a region between the ER and Golgi named vesicular tubular clusters (VTCs) or the ER-Golgi intermediate compartment (ERGIC) (3, 4). Although the boundaries between the ER, VTCs, and *cis*-Golgi are not definitively apparent, recent studies demonstrated that ER-resident proteins may be segregated in VTCs (5), and transported back to the ER by COPI vesicles via the retrograde pathway (6, 7). The transport of secretory proteins is presumably accomplished through the movement of VTCs from the cell periphery to perinuclear regions, and their subsequent fusion with the *cis*-Golgi network (8). For the formation and maturation of VTCs, numerous proteins are required including small GTP-binding proteins rab1 and rab2, SNAP receptors (SNAREs), and tethering proteins such as GM130 and p115 (9). Tethering proteins are peripheral membrane proteins with coiled–coil structures, which mediate the tethering of transport vesicles to the target membranes (10).

COPII consists of two protein complexes, Sec23p-Sec24p and Sec13p-Sec31p, and a low molecular weight GTP binding protein, Sar1p (11). Although it is well established that the GTP-hydrolysis cycle regulates the formation and uncoating of COPII vesicles (1, 2), other mechanisms may also work to modulate these processes. Recent studies demonstrated that phospholipid metabolism is crucial for the fission of membranes from the Golgi (12) and plasma membrane (13). It was hypothesized that conversion of lysophosphatidic acid, an inverted cone-shaped lipid, to phosphatidic acid, a cone-shaped lipid, induces fission of membranes (12, 13). Similar changes in lipids may occur in other membrane systems.

We previously reported a novel protein, p125, which interacts with mouse Sec23p and exhibits significant homology with phosphatidic acid-preferring phospholipase A_1 (14). When p125 was transiently expressed in cultured cells, it colocalized with ERGIC53, a well-known marker protein for ERGIC (VTCs) (15) and/or β-COP, a subunit of COPI located in the *cis*-Golgi (16), whereas its overexpression disrupted the localization of these proteins. In the present study we determined the regions of p125 that are responsible for Sec23p-binding and its localization.

[1] To whom correspondence should be addressed. Fax: +81-426-76-8866. E-mail: tani@ls.toyaku.ac.jp.

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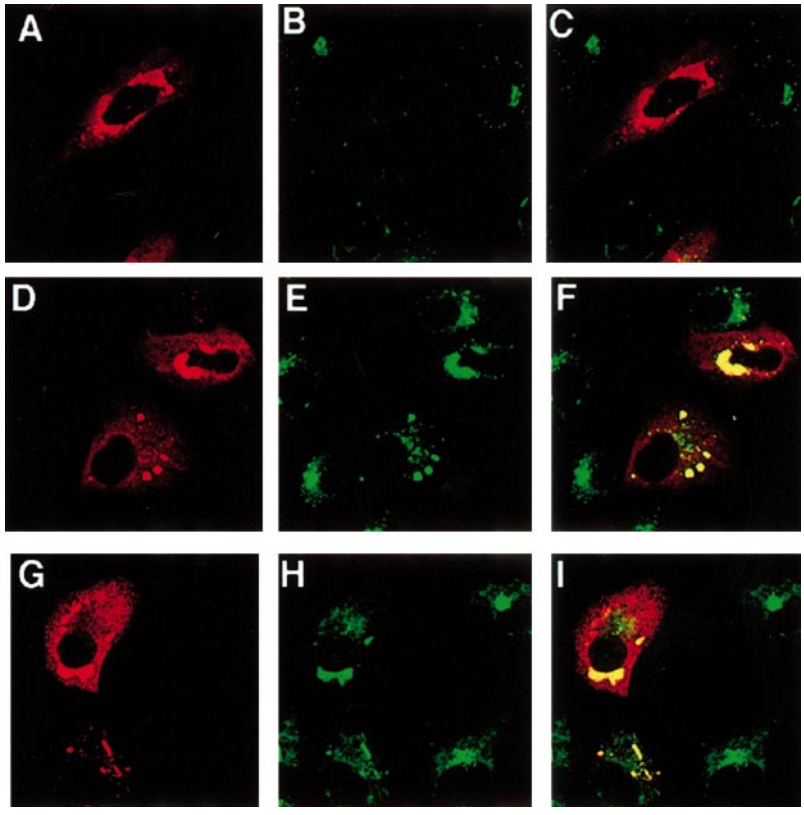

FIG. 1. Overexpression of p125 causes dispersion of syntaxin 5, but not p115 or GM130. Vero cells were transfected with the plasmid for FLAG-p125. At 24 h after transfection, the cells were fixed and double-stained with anti-FLAG (A, D, and G), and anti-syntaxin 5 (B), anti-p115 (E), or anti-GM130 (H). Merged images are shown on the right (C, F, and I).

MATERIALS AND METHODS

Antibodies. Polyclonal antibodies against p125, mouse Sec23p, and human Sec24p were prepared as described (14, 17). A monoclonal anti-ERGIC53 antibody was prepared as described (15). Polyclonal antibodies against β-COP (anti-EAGE) (16) and *Escherichia coli*-expressed syntaxin 5 were raised in this laboratory. Monoclonal anti-GM130 and anti-p115 antibodies were obtained from Transduction Laboratories.

Plasmids, cell culture, and transfection. Mammalian expression plasmids pEBG (18) and pFLAG-CMV-2 (Eastman Kodak Co.) were used to express proteins fused with the N-terminal GST and N-terminal FLAG epitope, respectively. For the construction of a series of truncated p125, cDNAs encoding partial sequences of p125 were amplified by the polymerase chain reaction and inserted into pEBG. Cell culture and transfection of plasmids were performed as described previously (14).

In vitro binding assay. The preparation of cell lysates and binding assay were performed as described previously (14).

Immunofluorescence microscopy. Confocal microscopic analysis was performed with an Olympus Fluoview 300 laser scanning microscope.

RESULTS

Colocalization of expressed p125 with p115 and GM130. In a previous study we showed that FLAG-tagged p125, when expressed at low levels, colocalizes with a VTCs/ERGIC marker, ERGIC53, and a *cis*-Golgi marker, β-COP, and its overexpression causes dispersion of these proteins (14). In the present study we first examined the localization of other proteins between the ER and Golgi in p125-expressing cells. As shown in Figs. 1A, 1D, and 1G, when expressed at high levels, FLAG-p125 was observed throughout the cells, but still principally localized in the perinuclear region. In these cells, dispersed patterns were observed for syntaxin 5 (Fig. 1B), which is normally observed in the perinuclear region. Interestingly, staining for p115, a protein located in VTCs and involved in vesicle tethering (19, 20), was not dispersed upon the overexpression of p125, and the perinuclear localization of FLAG-p125 principally overlapped with that of p115 (Figs. 1D, 1E, and 1F). In addition to p115, GM130, another tethering protein (19), also markedly colocalized with the expressed FLAG-p125 (Figs. 1G, 1H, and 1I). Similar results were obtained when p125 without a tag was expressed (data not shown), indicating that these phenotypes of FLAG-p125-expressing cells are not artifacts due to the addition of FLAG-tag to p125. These results suggest that expressed p125 mainly targets to compartments containing tethering proteins such as p115 and GM130.

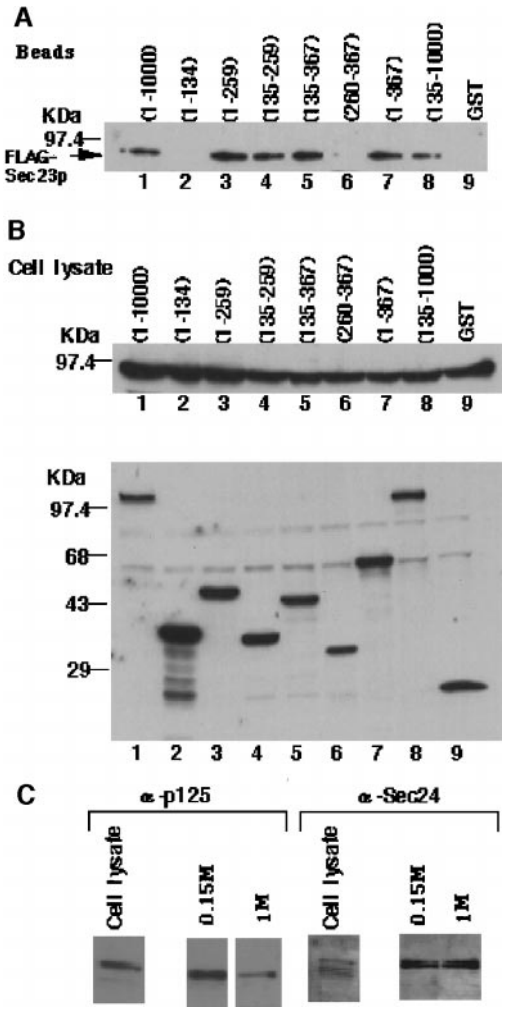

FIG. 2. The proline-rich region of p125 interacts with Sec23p. (A) The expression plasmid for GST, GST-full-length p125 (residues 1–1000), or GST-truncated p125 was transfected with the plasmid for FLAG-tagged Sec23p into 293T cells. At 24 h after transfection, the cells were lysed in lysis buffer containing 0.15 M KCl, and the lysates were incubated with glutathione beads. The bound proteins were analyzed by SDS–polyacrylamide gel electrophoresis and immunoblotting with anti-FLAG. The numbers in parentheses represent the residue numbers of p125. (B) For estimation of the amounts of expressed proteins in the lysates, 4% of the lysates was separated and immunoblotted with anti-FLAG (upper panel) or anti-GST (lower panel). (C) The expression plasmid for GST-Sec23p was transfected into 293T cells, and then the cell lysates were incubated with glutathione beads. The beads were washed with lysis buffer containing 0.15 M or 1 M KCl three times. Bound endogenous p125 and Sec24p were detected by immunostaining with anti-p125 and anti-Sec24p antibodies, respectively. For comparison, 2% of the lysates was analyzed. Several bands detected with the anti-Sec24p antibody may represent multi-species of the Sec24p family (26).

The proline-rich region of p125 binds to Sec23p. We demonstrated that the N-terminal 367 amino acids of p125 interact with Sec23p (14). To define further the region of p125 involved in the interaction with Sec23p, we created a series of truncated p125 proteins. FLAG-tagged Sec23p and a series of truncated p125 proteins fused to GST were coexpressed in 293T cells, and then pull-down assays were performed. GST version of p125 was used in this and further experiments because several FLAG-tagged truncation mutants were not efficiently expressed in cultured cells for unknown reasons. As shown in Fig. 2A, FLAG-tagged Sec23p was coprecipitated with p125 (residues 1–259), p125 (residues 135–259), p125 (residues 135–367), p125 (residues 1–367), and p125 (residues 135–1000), but not with p125 (residues 1–134) or p125 (residues 260–367). Although the levels of expression of GST-p125 mutants were somewhat different (Fig. 2B, lower panel), the most highly expressed mutant, p125 (residues 1–134), did not bind to FLAG-tagged Sec23p, suggesting binding specificity. We concluded from these results that a region comprising residues 135–259 of p125 is responsible for the binding to Sec23p. This region is rich in proline-residues (28% of the residues), this value being about threefold higher than the proline content of p125 (8.6%).

It is known that the mammalian and yeast Sec23p–Sec24p complexes are stable in the presence of high salt (17, 21). We therefore examined whether or not p125 also binds tightly to Sec23p. For this purpose, GST-Sec23p was expressed in 293T cells, and then pull-down assays were performed for endogenous p125 and Sec24p. As shown in Fig. 2C, the binding of GST-Sec23p to p125 was abrogated by 1 M KCl, whereas that of GST-Sec23p to Sec24p was resistant. These results suggest that the interaction between Sec23p and p125 is not as strong as that between Sec23p and Sec24p. It seems that p125 does not form a stable complex with Sec23p but rather interacts transiently with Sec23p.

Both the proline-rich and phospholipase A_1 homology regions are required for the perinuclear localization of p125. We next examined which region of p125 directs its perinuclear localization. For this purpose, a series of truncated forms of p125 (residues 1–134, residues 1–259, residues 135–259, or residues 273–1000) were expressed as GST fusion proteins in cultured cells. Residues 273–1000 correspond to the phospholipase A_1 homology region. As shown in Figs. 3A, 3C, 3E, and 3G, all mutants, irrespective of their expression levels, were distributed throughout the cytoplasm and did not localize to the perinuclear region. These results indicate that neither the proline-rich region (residues 135–259) responsible for the binding to Sec23p, nor the phospholipase A_1 homology region (residues 273–1000) is sufficient for the correct targeting of p125. Interestingly, expression of p125 (residues 1–134) (Fig. 3B) or p125 (residues 1–259) (Fig. 3D) elicited dispersion of ERGIC53. Dispersion patterns were also observed for

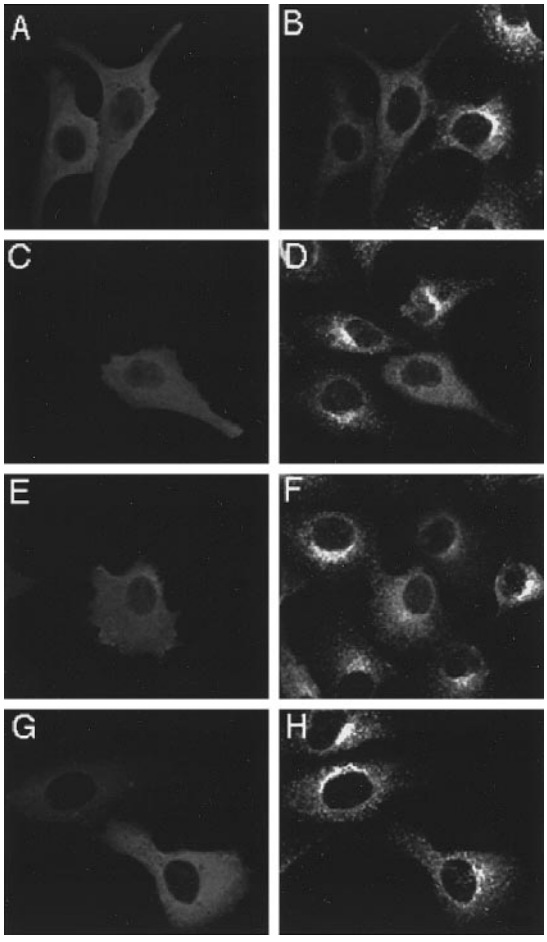

FIG. 3. Neither the proline-rich region nor the phospholipase A_1 homology region is sufficient for the targeting of p125 to the perinuclear region. The expression plasmid for p125 (residues 1–134) (A and B), p125 (residues 1–259) (C and D), p125 (residues 135–259) (E and F), or p125 (residues 273–1000) (G and H) was transfected into Vero cells. At 24 h after transfection, the cells were fixed and double-stained with anti-GST (A, C, E, and G) and anti-ERGIC53 (B, D, F, and H).

β-COP (data not shown). These results suggest that the N-terminal 134 residues have the ability to disrupt organelles in the early secretory pathway.

To determine a minimum region required for the perinuclear localization of p125, we expressed p125 (residues 135–1000) comprising both the proline-rich and phospholipase A_1 homology regions. As shown in Fig. 4, expressed p125 markedly colocalized with p115, as observed for full-length p125. In the case of expression of p125 (residues 135–1000), ERGIC53 was not dispersed, but rather colocalized with expressed p125. This may be due to the low level expression of the mutant or the lack of the N-terminal region (residues 1–134) having the activity to disrupt membrane structures.

DISCUSSION

p125 is a novel Sec23p-interacting protein containing a proline-rich region and a phosphatidic acid-preferring phospholipase A_1 homology region (14). In the present study we first examined the localization of several proteins between the ER and Golgi apparatus in p125-expressing cells. We found that tethering proteins such as p115 and GM130 principally colocalized with expressed p125. It should be noted that the phenotype of p125-expressing cells is unique. Overexpression of syntaxin 5, a Golgi SNARE, or syntaxin 18, an ER SNARE, causes not only redistribution of ER-Golgi recycling proteins such as β-COP and ERGIC53 but also dispersion of a tethering protein, GM130 (22). In contrast, expressed p125 appeared to stabilize and "solidify" compartments containing tethering proteins in the perinuclear region.

Our results suggest that the proline-rich region (residues 135–259) is involved in the binding to Sec23p. In this region there is the sequence ^{208}GPPAHPPPSGP^{218}, which is similar to the consensus sequence of the SH3 binding domain (XPXXPPPZXP, where X and Z represent any amino acid residue and a hydrophobic amino acid residue, respectively) (23). We examined whether a peptide corresponding to residues 208–218 of p125 affects the binding of p125 to Sec23p in a pull-down assay. However, FLAG-tagged Sec23p was coprecipitated with GST-p125 even in the presence of this peptide (data not shown). Further studies are required to specify the amino acid residues of p125 involved in the binding to Sec23p.

One of the interesting results in this study is that both the proline-rich and phospholipase A_1 homology regions are required for the perinuclear localization of p125. The phosphatidic acid-preferring phospholipase A_1 identified by Higgs and Glomset (24) does not possess a proline-rich region. Recently, the presence of another putative phospholipase A_1 encoded by KIAA0725 was reported. The KIAA0725 protein, like phosphatidic phospholipase A_1, has no proline-rich region (25). It is likely that these three proteins (phosphatidic acid-preferring phospholipase A_1, p125, and KIAA0725 protein) constitute a novel phospholipase family. We assume that the phospholipase domain of p125 may play a role in the anchorage to membranes, and that the extra proline-rich region only present in p125 may be responsible for membrane specificity.

The binding between Sec23p and p125 is not tight compared with that between Sec23p and Sec24p. Consistent with this, gel filtration analysis of bovine brain cytosol showed that Sec24p was exactly coeluted with Sec23p, whereas p125 was not (data not shown). These results suggest that p125 interacts transiently with Sec23p. This transient interaction may contribute to the targeting of p125 to compartments containing teth-

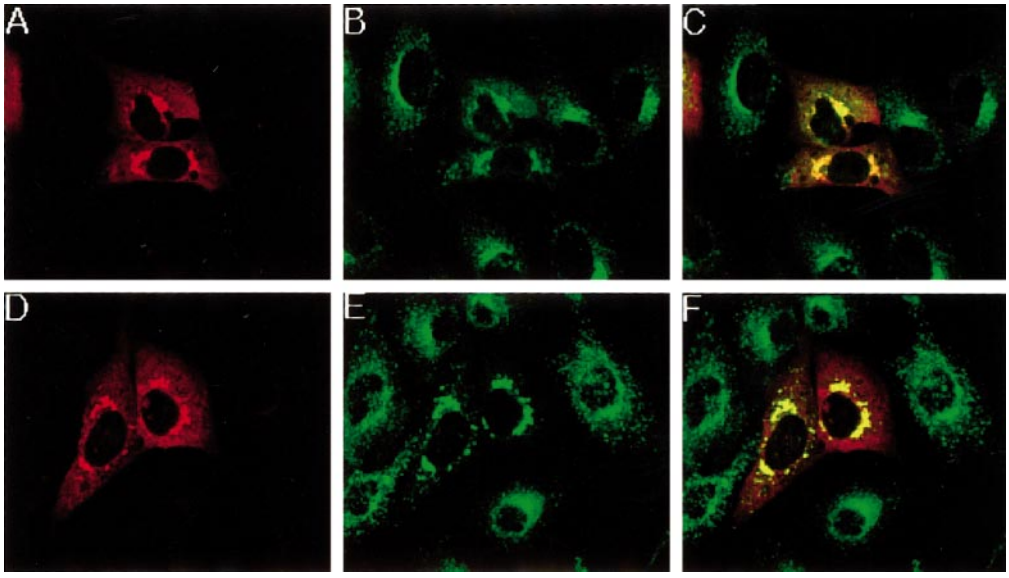

FIG. 4. Expressed p125 (residues 135–1000) targets to the compartments containing p115 and ERGIC53. Vero cells were transfected with the plasmid for GST-p125 (residues 135–1000). At 24 h after transfection, the cells were fixed and double-stained with anti-GST (A and D) and anti-p115 (B) or anti-ERGIC53 (E). Merged images are shown on the right (C and F).

ering proteins. In those compartments p125 may play a role in the formation and/or maturation of VTCs.

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