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Physicochemical Properties of α-Taxilin as a Putative Tethering Factor

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Summary

α-Taxilin is a member of the taxilin family, a binding partner of the syntaxin family involved in intracellular vesicle traffic. The taxilin family members share a long coiled-coil structure, which is homologous to that of Uso1, a yeast homologue of p115. It has been revealed that a parallel homodimerization of p115, an elongated polypeptide, is required for tethering of the transport vesicles to the acceptor membrane. In this study we examined whether α-taxilin has similar biochemical properties to p115. Gel filtration and sedimentation analyses suggest that α-taxilin is an elongated polypeptide containing a long coiled-coil structure. A pull-down assay revealed that His_6-α-taxilin binds to GST-α-taxilin, and not to GST in dose-dependent and saturable manners. α-Taxilin formed a parallel homodimer through at least two independent regions. Together, α-taxilin may be involved in tethering of the transport vesicles to the acceptor membranes in a similar way to p115.

Key Words: α-taxilin, intracellular vesicle traffic, SNARE, syntaxin

Introduction

Intracellular vesicle traffic is an essential process for the establishment and maintenance of organelle identity, biosynthetic transport, formation of cell polarity, and cell division and motility[1]. Vesicles derived from a donor compartment are fused with the specific acceptor membrane, resulting in their cargo delivery to the correct destination[1]. To achieve the necessary accuracy, intracellular vesicle traffic is regulated through a strict succession of highly interdependent four processes including vesicle budding, transport, tethering, and docking/fusion ones[1]. Among them, tethering process is characterized by the specific tethering of vesicles to the acceptor membrane[2]. Docking/fusion process is characterized by the specific assembly that occurs between members of the SNARE machinery[3]. The tethering process precedes the docking/fusion process. Both the processes are temporally and spatially regulated by a variety of molecules including proteins, lipids, and ions, and contribute to targeting specificity[2,3].

The SNARE machinery is composed of v- and t-SNAREs, all of which contain the SNARE motif, a coiled-coil structure with a central ionic layer. A unitary v-SNARE resided on vesicles pairs with its cog-

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nate t-SNAREs resided on the acceptor membrane, forming a biochemically stable *trans*-SNARE complex[3]. The *trans*-SNARE complex bridges vesicles close to the acceptor membrane and the two lipids bilayers are brought into close apposition and subsequently fusion occurs. v-and t-SNAREs are composed of the VAMP family, and the syntaxin and SNAP-25 families, respectively[4]. Each family is composed of numerous members, each of which is localized on a distinct subcellular membrane component. The distinct localization and specific assembly of v- and t-SNAREs contribute to specificity on intracellular vesicle traffic[4]. It is thought that among v- and t-SNAREs, the syntaxin family plays a pivotal role in the formation of the *trans*-SNARE complex[5].

Tethering process is mediated by tethering factors, which are divided into the following two groups: one is a group of soluble elongated polypeptides containing a long coiled-coil structure and the other is a group of multisubunit complexes[2]. Each tethering factor is recruited to a distinct subcellular membrane component and subsequently compartment specific tethering complexes are formed by the assembly of a specific set of tethering factors, resulting in tethering of vesicles to the target membrane[2]. Particularly, p115, a soluble elongated polypeptide containing a long coiled-coil structure, is recruited to COPI vesicles by Rab1 small GTPase[6-8]. The COPI vesicles are required for retrograde transport from the cis-Golgi back to the ER[9]. On the COPI vesicles, p115 directly and independently interacts with syntaxin-5 and GOS-28, and promotes their association[10]. The homodimerization of p115 providing a physical link between syntaxin-5 and GOS-28 is required for the formation of the *trans*-SNARE complexes containing syntaxin-5[10]. Moreover, the homodimerization is also required for the formation of the tethering complexes containing p115 such as a tethering complex including p115, Giantin, and GM130, which is formed in tethering of the COPI vesicles to Golgi membranes[10].

We have previously identified a novel syntaxin-binding protein family and named it the taxilin family, which is composed of α-taxilin, β-taxilin/MDP77, and γ-taxilin[11-13]. The taxilin family members share a long coiled-coil structure homologous to that of Uso1, a yeast homologue of p115[6,11]. α- and γ-Taxilins are ubiquitously expressed in various tissues but β-taxilin/MDP77 is abundantly expressed in the skeletal muscle and heart[12]. Accumulating evidence suggest that the taxilin family has diverse functions[11,13-15]. We have reported that α-taxilin may be involved in intracellular vesicle traffic, and transcriptional and translational processes through its interaction with the syntaxin family members and nascent polypeptide-associated complex, respectively[11,14]. α-Taxilin interacts with the syntaxin family members resided on the plasma membrane such as syntaxin-1a, -3, and -4[11]. α-Taxilin interacts with syntaxin-1a free from VAMP2 and SNAP-25, and not with that forming the *trans*-SNARE complex with VAMP2 and SNAP-25[16]. Taken together with the fact that α-taxilin contains a coiled-coil structure homologous to that of Uso1, a yeast tethering factor, we think that before the formation of the *trans*-SNARE complex, α-taxilin may function as a tethering factor in intracellular vesicle traffic. Then, to provide insights into a mode of action of α-taxilin in intracellular vesicle traffic, we here examined whether α-taxilin has also biochemical properties essential for the functions of Uso1 and p115 as a tethering factor in intracellular vesicle traffic.

Materials and Methods

Materials and Chemicals

GST- and His_6-α-taxilins were prepared as described previously[16]. Anti-His_6 and anti-GFP antibodies were from MBL (Nagoya, Japan). Other materials were from commercial sources.

Superdex 200 Gel filtration of α-taxilin

His_6-α-taxilin (0.5 nmol) in 0.1 ml of Buffer A (20 mM Tris/HCl at pH 7.4, 1M NaCl, 1 mM EDTA, 1 mM dithiothreitol, and 0.1% Triton X-100) was applied to a Superdex 200 HR 10/30 column (1 × 30 cm) (GE Healthcare, Little Chalfont, Backinghamshire, England) equilibrated with 48 ml of Buffer A at 0.25 ml/min. Fractions of 0.5 ml each were collected. An aliquot (20 ∝l) of each fraction was subjected to SDS-PAGE followed by silver staining or Western blotting with the anti-His_6 antibody. Molecular size standards used were α2-macroglobulin (Mr = 770,000), catalase (Mr = 245,000), and bovine serum albumin (Mr = 66,000). We failed to perform the same experiment by use of

Buffer B (20 mM Tris/HCl at pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, and 0.1% Triton X-100) instead of Buffer A because His_6-α-taxilin could not be eluted from the column due to its complete absorption into the column.

Velocity Sedimentation of α-taxilin

The indicated samples were separately layered onto a 5-10% linear glycerol gradient in Buffer B. Centrifugation was performed at 238,000 × *g* for 5.5 h at 4 ℃. Fractions of 0.475 ml each were collected from the top to the bottom. An aliquot of each fraction was subjected to SDS-PAGE followed by silver staining or Western blotting with the indicated antibodies. S value standards used were catalase (S = 11.4) and bovine serum albumin (S = 4.6).

Plasmid construction

Standard recombinant DNA techniques were used to construct the following expression constructs: pEGFP/α-taxilin (full length), pEGFP/α-taxilin$_{1\text{-}199}$ (amino acids 1-199), pEGFP/α-taxilin$_{1\text{-}256}$ (amino acids 1-256), pEGFP/α-taxilin$_{1\text{-}361}$ (amino acids 1-361), pEGFP/α-taxilin$_{200\text{-}546}$ (amino acids 200-546), pEGFP/α-taxilin$_{257\text{-}546}$ (amino acids 257-546), pEGFP/α-taxilin$_{362\text{-}546}$ (amino acids 362-546), pcDNA-myc/α-taxilin (full length), pcDNA-myc/α-taxilin$_{1\text{-}361}$ (amino acids 1-361), pcDNA-myc/α-taxilin$_{200\text{-}546}$ (amino acids 200-546), and pcDNA-myc/α-taxilin$_{257\text{-}546}$ (amino acids 257-546). The constructions were done by inserting the fragments generated by PCR or by restrict digestion into the vectors. The entire PCR products were sequenced and the structures of all plasmids were confirmed by restriction analysis.

Transfection and Immunoprecipitation

HeLa cells were cultured at 37 ℃ in Dulbecco's modified Eagle's medium containing 10% foetal calf serum (Invitrogen Corp., San Diego, CA, USA), 100 ∝g/ml streptomycin, and 100 units/ml penicillin. Transient transfection of the indicated expression plasmids into HeLa cells was performed by use of Lipofectamine™ 2000 reagent (Invitrogen Corp.) according to the manufacturer's instructions. The transfected cells were harvested at 24 h post-transfection. Harvested cells from a 100-mm dish were lysed with 2.4 ml of Buffer B containing 10 ∝M *p*-amidinophenylmethanesulphonyl and 10 ∝M leupeptin on ice for 1 h. The lysate was centrifuged at 15,000 × *g* for 10 min at 4 ℃ and the supernatant was used as a Triton X-100-solubule fraction. To immunoprecipitate myc-α-taxilin, the indicated samples were incubated with the anti-myc monoclonal antibody coupled to protein G-Sepharose beads (GE Healthcare). After intensively washing the beads, proteins bound to the beads were eluted with Laemmli's sample buffer. An aliquot (5% for detecting proteins fused with myc; 50% for detecting proteins fused with EGFP) of the eluate was subjected to SDS-PAGE followed by Western blotting with the indicated antibodies.

Pull-down Assay

GST or GST-α-taxilin (50 pmol each) was immobilized on 20 ∝l of glutathione Sepharose 4B beads (GE Healthcare) equilibrated with Buffer A. After washing the beads with the same buffer, the beads were equilibrated with Buffer B and incubated with various concentrations of His_6-α-taxilin in 0.2 ml of Buffer B for 2 h at 4 ℃. After intensively washing the beads, proteins bound to the beads were eluted with the Laemmli's sample buffer. An aliquot (20%) of the eluate was subjected to SDS-PAGE followed by Western blotting with the anti-His_6 antibody. The amounts of His_6-α-taxilin bound to the beads were quantified by Fuji Film image gauge V3.4 analysis software (Fuji Film, Tokyo, Japan) with His_6-α-taxilin as a standard.

Other Procedures

Protein concentrations were determined with bovine serum albumin as a reference protein as described[17]. SDS-PAGE was performed as described[18]. Western blotting was performed using ECLplus immunoblotting detection system (GE Healthcare) according to the manufacturer's instructions.

Results

α-Taxilin as an elongated polypeptide

Biochemical studies have revealed that p115 is an elongated homodimeric molecule[7]. Moreover, it has been shown by use of quick-freeze, deep-etch, rotary shadowing electron microscopy that p115 dimerizes via the coiled-coil region[6]. We first examined whether α-taxilin is an elongated polypeptide. His_6-α-taxilin was subjected to Superdex 200 column chromatography.

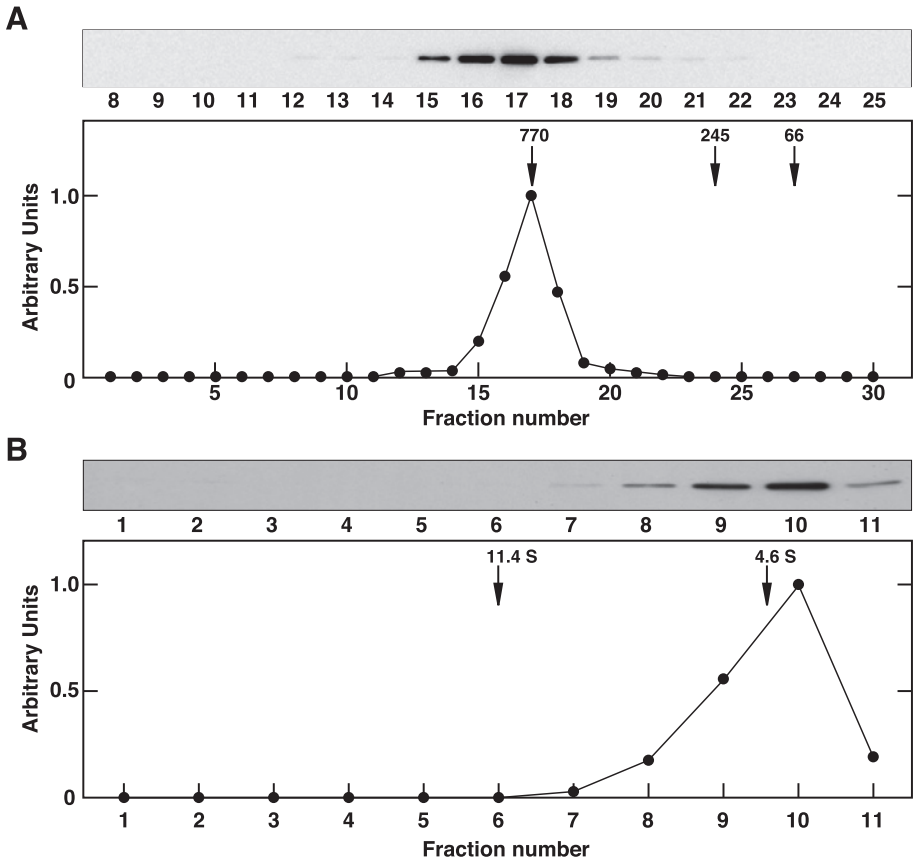

Figure 1 Superdex 200 gel filtration and velocity sedimentation of α-taxilin. **A**, Superdex 200 gel filtration of α-taxilin. His_6-α-taxilin was chromatographed on a Superdex 200 column. An aliquot of each fraction was subjected to SDS-PAGE followed by Western blotting with the anti-His_6 antibody. Upper panel, Western blotting; lower panel, elution profile. (●), His_6-α-taxilin. **B**, velocity sedimentation of α-taxilin. His_6-α-taxilin (0.5 nmol) was sedimented through a glycerol gradient. An aliquot (20 ∝ l) of each fraction was subjected to SDS-PAGE followed by Western blotting with the anti-His_6 antibody. Upper panel, Western blotting; lower panel, elution profile. (●), His_6-α-taxilin. The results shown are representative of three independent experiments.

His_6-α-taxilin appeared as a single peak at the position corresponding to a globular protein with a molecular mass of about 770 kDa (Fig. 1A). Since a calculated molecular weight of α-taxilin is 61,890, the result suggests that His_6-α-taxilin forms a homo-oligomer or that α-taxilin is a non-globular protein. Then, to assess two possibilities, we performed velocity sedimentation of His_6-α-taxilin. His_6-α-taxilin appeared as a single peak at the position with a molecular mass of about 50 kDa (Fig. 1B). Together, these results indicate that under our experimental conditions, a large part of His_6-α-taxilin is present as a monomer and that the molecular mass of the monomer His_6-α-taxilin estimated by the gel filtration analysis is much larger than its calculated molecular weight, implying that α-taxilin, like p115, is an elongated polypeptide containing a long coiled-coil structure.

Homodimerization of α-taxilin

Our data suggest that α-taxilin, unlike p115, is hardly present *in vitro* as a stable homodimer, but do not necessarily exclude the possibility that α-taxilin has the ability to form a homodimer. Then, we performed a pull-down assay by use of GST-and His_6-α-taxilins. When GST-α-taxilin immobilized on the beads was incubated with various concentrations of His_6-α-taxilin, His_6-α-taxilin bound to GST-α-taxilin, and not to GST in dose-dependent and saturable manners (Fig. 2 A). About 0.05 mol of His_6-α-taxilin maximally bound to one mol of GST-α-taxilin and the concentration of His_6-α-taxilin giving a half-maximal binding to GST-α-taxilin was about 0.25 ∝M.

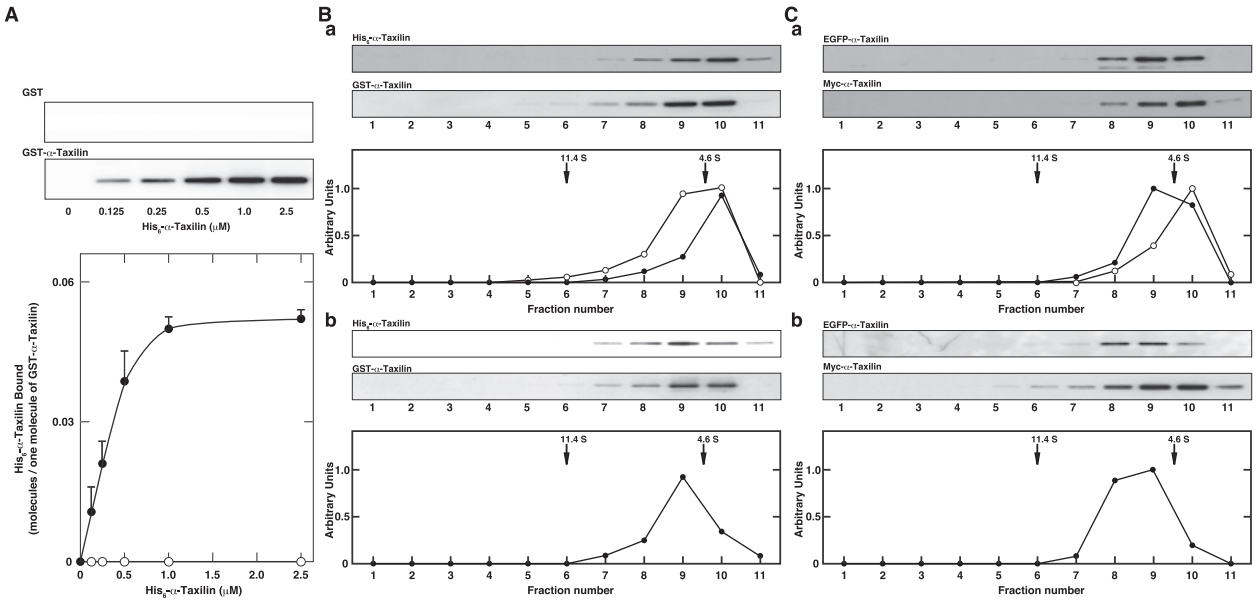

Figure 2 Homodimerization of α-taxilin. **A**, interaction between His_6- and GST-α-taxilins. Various concentrations of His_6-α-taxilin were incubated with GST or GST-α-taxilin immobilized on glutathione Sepharose 4B beads. Proteins bound to the beads were analyzed by Western blotting with the anti-His_6 antibody after SDS-PAGE. Upper panel, Western blotting; lower panel, quantification. (●), with GST-α-taxilin; (○), with GST. The values are expressed as means ± standard errors of three independent experiments. **B**, homodimerization of α-taxilin *in vitro*. A mixture of His_6- and GST-α-taxilins (1 ∝ M each) was sedimented through a glycerol gradient. In one set of experiments, an aliquot (20 ∝ l) of each fraction was subjected to SDS-PAGE followed by Western blotting with the anti-His_6 and anti-GST antibodies. In another set of experiments, an aliquot (0.1 ml) of each fraction was incubated with glutathione Sepharose 4B beads equilibrated with Buffer B. Proteins bound to the beads were analyzed by Western blotting with the anti-His_6 and anti-GST antibodies after SDS-PAGE. **a**, velocity sedimentation of His_6- and GST-α-taxilins. Upper panel, Western blotting; lower panel, elution profile. (●), His_6-α-taxilin; (○), GST-α-taxilin. **b**, complex formation of His_6-α-taxilin with GST-α-taxilin. Upper panel, Western blotting; lower panel, quantification. (●), His_6-α-taxilin pulled down with GST-α-taxilin. **C**, homodimerization of α-taxilin *in vivo*. The Triton X-100-soluble fraction (0.25 ml) of HeLa cells co-expressing EGFP- and myc-α-taxilins was sedimented through a glycerol gradient. In one set of experiments, an aliquot (20 ∝ l) of each fraction was subjected to SDS-PAGE followed by Western blotting with the anti-GFP and anti-myc antibodies. In another set of experiments, myc-α-taxilin was immunoprecipitated with the anti-myc monoclonal antibody from an aliquot (0.1 ml) of each fraction. An aliquot of the immunoprecipitate was subjected to SDS-PAGE followed by Western blotting with the anti-GFP and anti-myc antibodies. **a**, velocity sedimentation of EGFP- and myc-α-taxilins. Upper panel, Western blotting; lower panel, elution profile. (●), EGFP-α-taxilin; (○), myc-α-taxilin. **b**, complex formation of EGFP-α-taxilin with myc-α-taxilin. Upper panel, Western blotting; lower panel, quantification. (●), EGFP-α-taxilin co-immunoprecipitated with myc-α-taxilin. The results shown in **(B)** and **(C)** are representative of three independent experiments.

Next, we performed velocity sedimentation followed by a pull-down assay. A mixture of His_6- and GST-α-taxilins was subjected to the same glycerol density gradient ultracentrifugation as described above. His_6- and GST-α-taxilins appeared at the positions with a molecular mass of about 50 kDa and about 60 kDa, respectively (Fig. 2Ba). When each fraction was incubated with glutathione Sepharose 4B beads, His_6-α-taxilin pulled down with GST-α-taxilin appeared as a single peak in Fraction 9, which corresponded to the position with a molecular mass of about 110 kDa (Fig. 2Bb). These results indicate that α-taxilin form a homodimer *in vitro*. To verify the homodimerization of α-taxilin, we performed velocity sedimentation followed by an immunoprecipitation assay. First, the Triton X-100-soluble fraction of HeLa cells co-expressing EGFP- and myc-α-taxilins was subjected to the same glycerol density gradient ultracentrifugation as described above. EGFP- and myc-α-taxilins appeared at the positions with a molecular mass of about 70 kDa and about

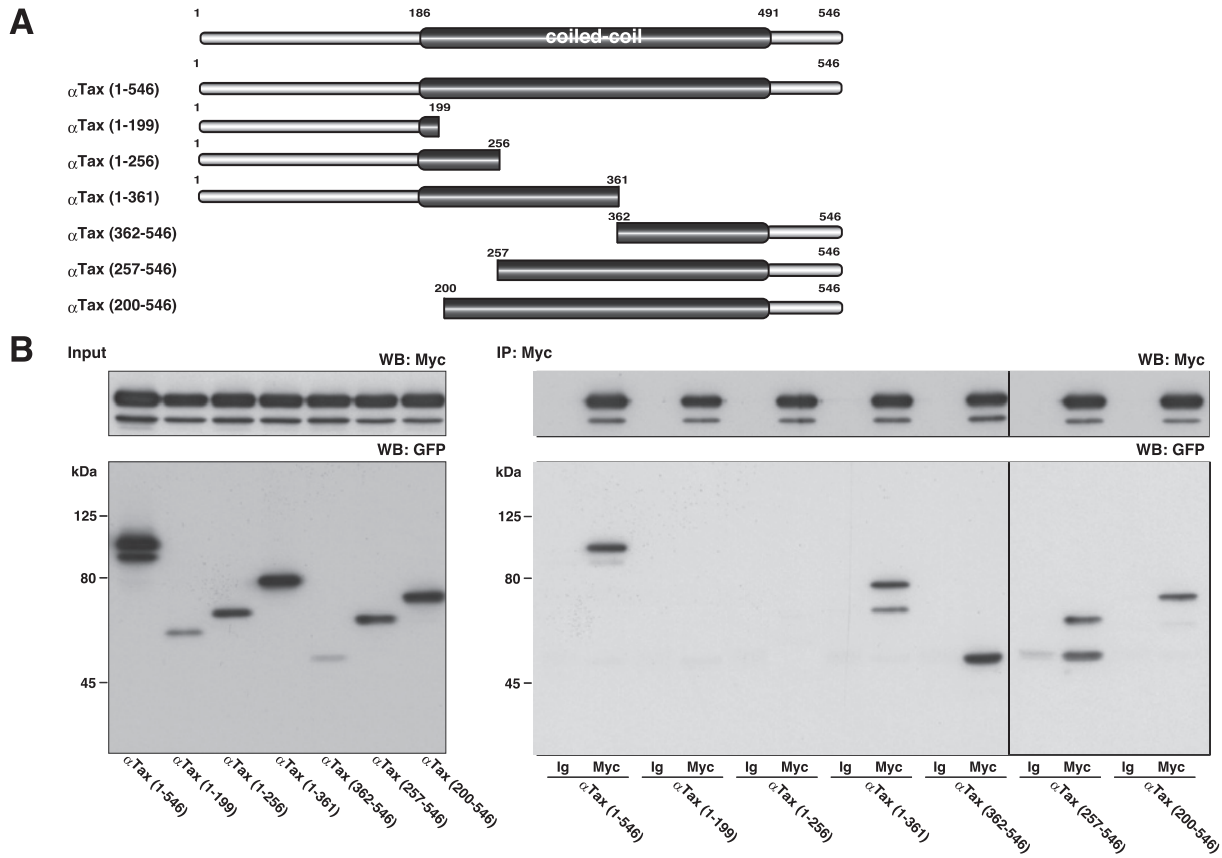

Figure 3 Responsible regions of α-taxilin for its homodimerization. Myc-α-taxilin was immunoprecipitated with the mouse immunoglobulin (Ig) or the anti-myc monoclonal antibody (Myc) from the Triton X-100-soluble fractions (0.1 ml) of HeLa cells co-expressing myc-α-taxilin and EGFP-α-taxilin or the indicated deletion mutants of α-taxilin fused with EGFP. Proteins bound to the beads were analyzed by Western blotting with two antibodies (indicated on the right side of each panel) after SDS-PAGE. Five percent of the amount used for immunoprecipitation was loaded as *input*. **A**, schematic representation of the predicted domain structure of α-taxilin and its deletion mutants. The deletion mutants are designated as αTax (X-X), where X-X represented amino acid residues. **B**, Western blotting. Left panels, input; right panels, immunoprecipitate. The results shown are representative of three independent experiments.

50 kDa, respectively (Fig. 2Ca). When the same experiments were performed by use of the Triton X-100-soluble fraction of HeLa cells not expressing any exogenous protein, endogenous α-taxilin appeared at the position with a molecular mass of about 50 kDa (data not shown). These results indicate that α-taxilin is abundantly present *in vivo* as a monomer. Second, myc-α-taxilin was immunoprecipitated with the anti-myc monoclonal antibody from each fraction. EGFP-α-taxilin co-immunoprecipitated with myc-α-taxilin appeared as a single peak in Fractions 8 and 9, which corresponded to the position with a molecular mass of 110-160 kDa (Fig. 2Cb). When the same experiments were performed by use of the mouse immunoglobulin instead of the anti-myc monoclonal antibody, neither myc- nor EGFP-α-taxilin was immunoprecipitated (data not shown). These results suggest that α-taxilin forms a homodimer *in vivo* and, moreover, raise the possibility that the homodimer of α-taxilin makes a complex with other molecules.

Responsible regions of α-taxilin for its homodimerization

To reveal responsible regions of α-taxilin for its homodimerization, we performed an immunoprecipitation assay by use of a series of deletion mutants of α-taxilin as shown in Fig. 3A. Myc-α-taxilin was immunoprecipitated from the Triton X-100-soluble fractions of HeLa cells co-expressing myc-α-taxilin and the indicated deletion mutants of α-taxilin fused with EGFP. Deletion

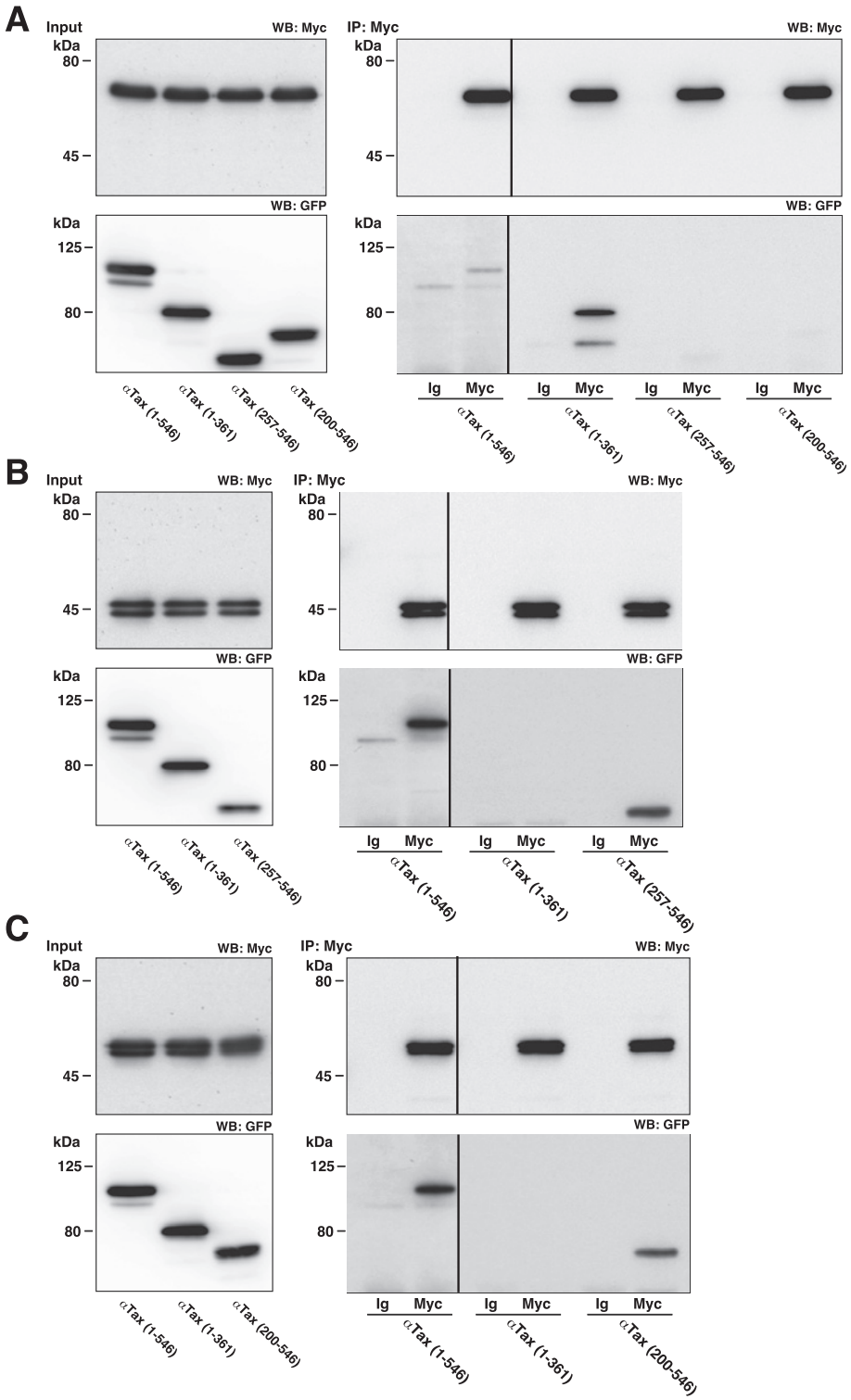

Figure 4 Interactions between deletion mutants of α-taxilin. Proteins fused with myc were immunoprecipitated with the mouse immunoglobulin (Ig) or the anti-myc monoclonal antibody (Myc) from the Triton X-100-soluble fractions (0.1 ml) of HeLa cells co-expressing myc-α-taxilin$_{1\text{-}361}$ **(A)**, myc-α-taxilin$_{257\text{-}546}$ **(B)**, or myc-α-taxilin$_{200\text{-}546}$ **(C)**, and EGFP-α-taxilin or the indicated deletion mutants of α-taxilin fused with EGFP. Proteins bound to the beads were analyzed by Western blotting with two antibodies (indicated on the right side of each panel) after SDS-PAGE. Five percent of the amount used for immunoprecipitation was loaded as *input*. The deletion mutants are designated as shown in Figure 3. Left panels, input; right panels, immunoprecipitate. The results shown are representative of three independent experiments.

mutants including the amino-terminal half and/or carboxyl-terminal half of the coiled-coil region were co-immunoprecipitated with myc-α-taxilin (Fig. 3B). These results indicate that α-taxilin forms a homodimer through its coiled-coil region and suggest that there are at least two independent regions responsible for the homodimerization.

Homodimerization of α-taxilin in parallel

To clarify whether α-taxilin, like p115, forms a parallel homodimer, we examined interactions between the deletion mutants by use of the same immunoprecipitation assay. Myc-α-taxilin$_{1\text{-}361}$ was immunoprecipitated from the Triton X-100-soluble fractions of HeLa cells co-expressing myc-α-taxilin$_{1\text{-}361}$ and the indicated deletion mutants of α-taxilin fused with EGFP. EGFP-α-taxilin$_{1\text{-}361}$, and not EGFP-α-taxilin$_{257\text{-}546}$ or EGFP-α-taxilin$_{200\text{-}546}$ was co-immunoprecipitated with myc-α-taxilin$_{1\text{-}361}$ (Fig. 4A). The same experiments were performed by use of or myc-α-taxilin$_{257\text{-}546}$ myc-α-taxilin$_{200\text{-}546}$ instead of myc-α-taxilin$_{1\text{-}361}$. EGFP-α-taxilin$_{257\text{-}546}$, and not EGFP-α-taxilin$_{1\text{-}361}$ was co-immunoprecipitated with myc-α-taxilin$_{257\text{-}546}$ (Fig. 4B). EGFP-α-taxilin$_{200\text{-}546}$, and not EGFP-α-taxilin$_{1\text{-}361}$ was co-immunoprecipitated with myc-α-taxilin$_{200\text{-}546}$ (Fig. 4C). Although we failed to perform the same experiments by use of myc-α-taxilin$_{362\text{-}546}$ due to its lower expression level, these results indicate that the amino-terminal half of the coiled-coil region of α-taxilin interacts with the same region, and not with the carboxyl-terminal half of its coiled-coil region and vice versa, suggesting that α-taxilin forms a parallel homodimer.

Discussion

We have previously shown the possibility that α-taxilin is involved in exocytosis through its interaction with the syntaxin family members localized on the plasma membranes[11]. Moreover, we have recently found that besides the cytoplasm, α-taxilin is localized on unknown intracellular organelles as a peripheral protein (unpublished data). In this study we found that α-taxilin may be an elongated polypeptide and that α-taxilin forms a parallel homodimer. These biochemical properties of α-taxilin were common to p115 and Uso1, both of which have a coiled-coil structure homologous to that of α-taxilin and function as a tethering factor in intracellular vesicle traffic[19]. We examined whether α-taxilin has SNARE motif or heptad repeat inside coiled-coil domain by SNARE Database (http://bioinformatics.mpibpc.mpg.de/snare/snareSubmitSequenceResultPage.jsp), but no relevant sequence was found. However, we have previously shown that α-taxilin binds to a SNARE motif (binding region: amino acids 191-239) of syntaxin3[11].

Taken together with the evidence that p115, like α-taxilin, interacts with the syntaxin family[10], although we have not yet identified vesicles containing α-taxilin, our present results strongly lead us to predict that α-taxilin may function as a tethering factor in transport of the vesicles to the plasma membrane in a similar way to p115.

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Author Contributions

Conception and design of the study: H.S.; Acquisition and analysis of data: S.H, H.S.; Drafting the manuscript and figures: S.H, H.S.

Competing interests

The authors declare no competing or financial interests.

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