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Isolation of a point-mutated p47 lacking binding affinity to p97ATPase

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ABSTRACT

p47, a p97-binding protein, functions in Golgi membrane fusion together with p97 and VCIP135, another p97-binding protein. We have succeeded in creating p47 with a point mutation, F253S, which lacks p97-binding affinity. p47 mapping experiments revealed that p47 had two p97-binding regions and the F253S mutation occurred in the first p97-binding site. p47(F253S) could not form a complex with p97 and did not caused any cisternal regrowth in an in vitro Golgi reassembly assay. In addition, mutation corresponding to the p47 F253S mutation in p37 and ufd1 also abolished their binding ability to p97.

Structured summary:
MINT-7987189, MINT-7987207, MINT-7987303: *p47* (uniprotkb:O35987) *binds* (MI:0407) to *p97* (uniprotkb:Q01853) by *pull down* (MI:0096)
MINT-7987226: *p97* (uniprotkb:P46462) *binds* (MI:0407) to *p47* (uniprotkb:O35987) by *pull down* (MI:0096)
MINT-7987348: *p97* (uniprotkb:P46462) *physically interacts* (MI:0915) with *Ufd1* (uniprotkb:P70362) by *pull down* (MI:0096)
MINT-7987264: *p97* (uniprotkb:P46462) and *p47* (uniprotkb:O35987) *bind* (MI:0407) by *competition binding* (MI:0405)
MINT-7987326: *p97* (uniprotkb:P46462) *binds* (MI:0407) to *p37* (uniprotkb:Q0KL01) by *pull down* (MI:0096)

1. Introduction

The Golgi apparatus plays a central role in intracellular membrane traffic. It undergoes dramatic transformation during the cell cycle [1]. It is fragmented into thousands of vesicles and short tubules and dispersed throughout the cytoplasm at mitosis [2,3]. During telophase, the mother cell is divided into two daughter cells, in each of which a Golgi apparatus rapidly reassembles from the fragments [3,4]. Experiments using an in vitro Golgi reassembly assay, which mimics the reassembly of Golgi stacks at the end of mitosis [5], showed that reassembly from membrane fragments requires at least two ATPases; N-ethylmaleimide-sensitive factor (NSF) and p97 [6]. The role of NSF has been well characterized, while much less is known about the mechanism of action of p97.

p97 has been shown to use two distinct cofactors for its membrane fusion function: p47 is specialized for the reassembly of organelles at the end of mitosis; p37 is required for organelles maintenance during interphase as well as for their reassembly during mitosis [7–9]. p97 also requires a deubiquitinating enzyme, VCIP135, for its membrane fusion function [10]. Since VCIP135 is another p97-binding protein, two p97-binding proteins are involved in each pathway. It is unclear how p97 utilizes two distinct p97-binding proteins: p47 and VCIP135 in the p97/p47/VCIP135pathway, and p37 and VCIP135 in the p97/p37/VCIP135 pathway. While investigating this point, we have succeeded in isolating a point-mutated p47 which lacks binding affinity to p97. p47 has two p97-binding sites, and the point mutation existed in the first binding site.

2. Materials and methods

2.1. Proteins and antibodies

Recombinant p97, p47, p37, Ufd1 and Npl4 were prepared as reported [7,9,11].

Abbreviations: NSF, N-ethylmaleimide-sensitive factor; VCIP135, VCP(p97)/p47 complex-interacting-protein, p135; His, six-histidine tag; GST, glutathione S-transferase

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To obtain GST-tagged deletion mutants of p47, the corresponding cDNAs with a stop codon were subcloned into pGEX4T-2 (Pharmacia). These cDNAs were also subcloned into pQE30 (QIAGEN). Recombinant p47 mutants were expressed in *E. coli* and purified according to manufacturer's instructions. Eluted samples were further purified using Superose 6 (Pharmacia).

To obtain a peptide, GSVKPKGAFKAKTGEGQKLGSTAPQVLNT [termed as BSU peptide (a peptide between SEP and UBX domains)], GST-tagged p47(244–270) was prepared in the same way as the other GST-tagged mutants. After purification with glutathione-beads, thrombin was added to the beads to cleave out the BSU peptide. The eluted BSU peptide was purified using Superose 12 (Pharmacia).

Point mutations in p47, p37, Ufd1 and BSU peptide were directly introduced by PCR reactions, using the Quick-change mutagenesis kit (Stratagene). All clones were verified by DNA sequencing.

Monoclonal antibodies to p97, GM130 and His-tag were purchased from Progen, BD Transduction and Qiagen, respectively.

2.2. Isolation of a p47 mutant which lacks binding affinity to p97

p47 mutants were generated and screened as described by Reddy et al. ([12]). The cDNA of rat p47(170–370) was amplified by a PCR reaction in which dATP was limited to 10% of the other dNTPs in order to generate PCR products with mutations randomly distributed throughout p47(170–370). The PCR products were subcloned into pGBKT7 vector. The ORF of rat p97 was also subcloned into pACT2 vector. Both plasmids were co-transformed into yeast strain AH109 and a yeast two-hybrid screen was carried out essentially according to manufacturer's protocol.

2.3. Binding experiments

Binding experiments were performed as described previously [9]. Briefly, GST-tagged proteins were immobilized to glutathione-beads, and were incubated with other proteins in buffer (0.15 M KCl, 20 mM HEPES, 1 mM ATP, 1 mM $MgCl_2$, 1 mM DTT, 5% glycerol, 0.1% Triton X-100, pH 7.4). After washing, the proteins bound on the beads were fractionated by SDS–PAGE and analyzed by Western blotting.

Golgi membranes were purified from rat liver ([13]) and washed with 1 M KCl as described previously ([10]). p47 or its mutant was incubated with salt-washed Golgi membranes in buffer (0.1 M KCl, 20 mM Tris, 0.2 M sucrose, pH 7.4) for 1 h on ice, and then the membranes were recovered by centrifugation.

2.4. ATPase assay

The ATP activity was measured using the technique described in Tagaya et al. [14]. For this assay, p97 was purified from rat liver as described previously [6]. Purified p97 was pre-incubated with full-length p47 or its deletion mutants on ice for 30 min before the assay. Samples were incubated in buffer (0.15 M KCl, 50 mM Tris–HCl, 2.5 mM $MgCl_2$, 1 mM ATP, 10μCi α-P^{32} ATP, 15% glycerol, pH 7.4) for 10–20 min at 37 C. One microliter of the reaction was then spotted in duplicate on polyethyleneimine cellulose thin layer plates (Polygram CEL 300PEI) and developed in 0.7 M LiCl, 1 M acetic aid. ATP and ADP spots were quantitated using a phosphorimager (Cyclone, Packard).

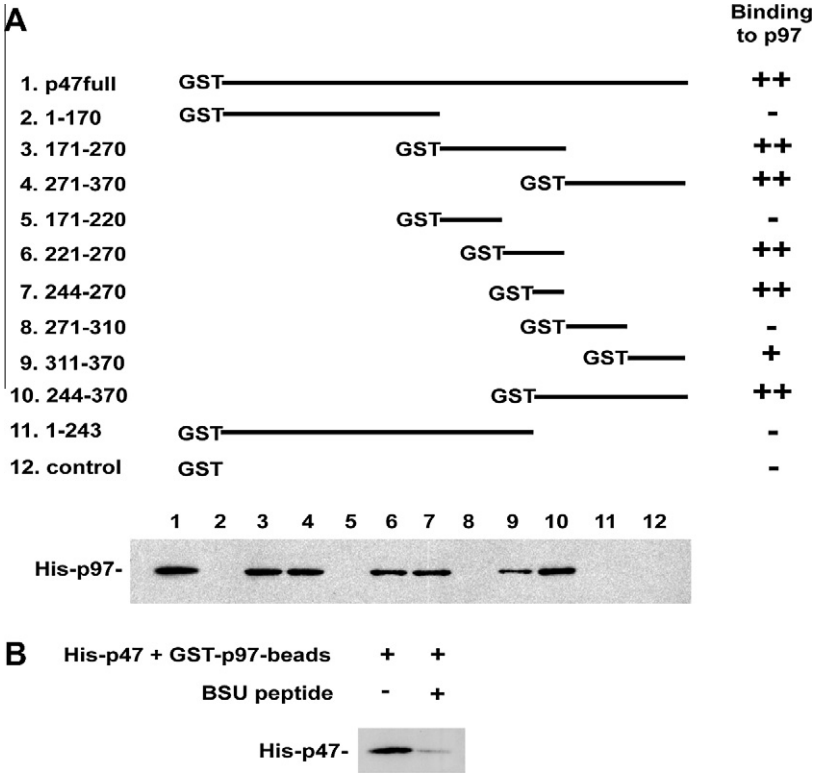

Fig. 1. Two p97-binding sites in p47. (A) Mapping of the p97-binding sites in p47. Each GST-tagged deletion mutant of p47 (1.5 μg) was immobilized to glutathione-beads. The beads were incubated with p97 (2 μg) in a buffer containing 0.1% Triton X-100. After washing, the bound p97 was fractionated by SDS-PAGE. Blots were probed with antibodies to the His tag on p97. (B) Stripping of p47 from the complex by BSU peptide. GST-tagged p97 (1.5 μg) that was immobilized to glutathione-beads was first incubated with p47 (2 μg). After removing unbound p47, BSU peptide (10 μg) was added into the beads. After washing, the bound proteins were fractionated by SDS–PAGE. Blots were probed with antibodies to the His tag.

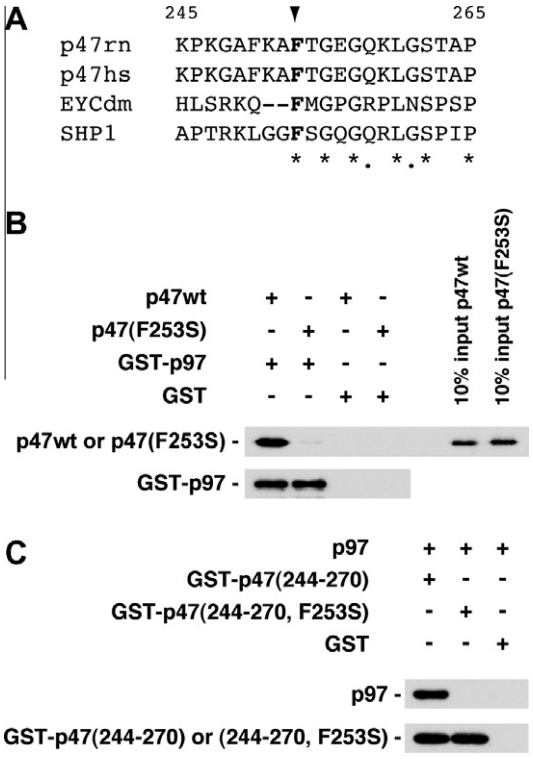

Fig. 2. Isolation of the p47(F253S) mutant. (A) Amino acid sequence alignment of p47 homologues (rat p47, human p47, *Drosophila* EYC and yeast SHP1). Identical residues between the homologues are marked with asterisks. The arrowhead indicates Phe-253 in p47. (B) GST-tagged p97 (0.8 μg) was immobilized to glutathione-beads. The beads were incubated with either His-tagged p47wt or p47(F253S) (0.05 μg) in a buffer containing 0.15 M KCl and 0.1% Triton X-100 on ice. The blots were probed with antibodies to p97 and His-tag. (C) Either GST-tagged p47(244–270) or p47(244–270, F253S) (0.1 μg) was immobilized to glutathione-beads. The beads were incubated with His-tagged p97 (0.6 μg) on ice. The blots were probed with antibodies to p97 and GST-tag.

2.5. In vitro Golgi reassembly assay

The in vitro Golgi reassembly assay was performed as reported previously [15]. All proteins added in this assay were prepared as recombinant proteins from *E. coli*. The length of cisternae was measured by an intersection method [5].

3. Results and discussion

3.1. Mapping of p97-binding sites in p47

We previously carried out a rough mapping experiments of p47 and showed that p47(171–270) and p47(271–370) fragments independently bound to p97 [10]. Bruderer et al. [16] found that the [246–266] portion of our first p97-binding p47 fragment could bind to p97 in a mapping experiment of ufd1, another p97-binding protein. Although these rough or fragmented mapping experiments of p47 were reported, comprehensive and detailed p47 mapping data does not exist, and hence we performed such an experiment as shown in Fig. 1A. p47(171–270) bound to p97 (lane 3) as we previously observed. A C-terminal half fragment (221–270) and a quarter fragment (244–270), which almost corresponds to the p97-binding fragment reported by Bruderer et al., bound to p97 (lanes 6 and 7), but its N-terminal fragment (171–220) could not bind p97 (lane 5). p47(171–243), which covers the SEP domain, neither bound to p97 (data not shown). p47(271–370) and its C-terminal half (311–370), which covers the UBX domain, bound to p97 (lanes 4 and 9), but its N-terminal half (271–370) did not (lane 8). Summing up these results, p97-binding regions in p47 were narrowed down to two sites [(244–270) and (311–370)]. The significance of the existence of two distinct p97–p47 binding interactions is thought to increase the conformational diversity of the complex; it could result in transient or unstable complexes that use only one p97–p47 binding interaction. Base on this idea, we previously succeeded in the identification of VCIP135, p97/p47 complex-interacting protein [10].

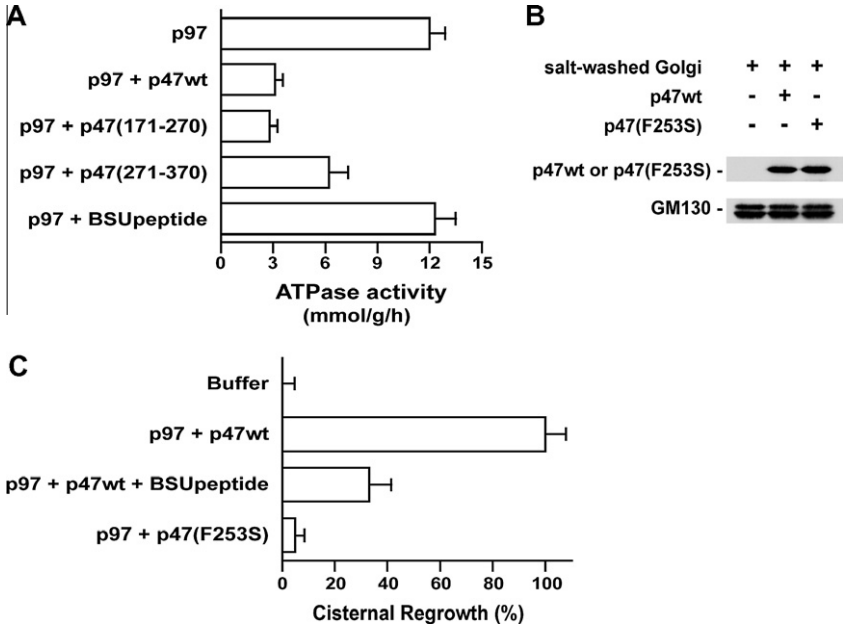

Fig. 3. Complex formation between p97 and p47 is essential for p97/p47/VCIP135-mediated Golgi membrane fusion. (A) Inhibition of p97 ATPase by p47 fragments. Purified p97 (0.5 μg) was pre-incubated with 1 μg of full-length p47 or p47 fragments containing the p97-binding sites. ATPase activities were measured using α-P^{32} ATP. (B) Either His-tagged p47wt or p47(F253S) (1.5 μg) was incubated with 1 M KCl-washed Golgi membranes (10 μg) and then the membranes were isolated by centrifugation, followed by Western blotting. Blots were probed with antibodies to the His-tag and GM130. (C) Mitotic Golgi membranes were incubated with the indicated components at 37 °C for 60 min: p97 (40 μg /ml), p47wt (20 μg /ml), p47(F253S) (20 μg /ml) and BSU peptide (20 μg /ml). Results are presented as the percentage of cisternal regrowth + SD (n = 4): 0% represents the buffer (22.5% in cisternal membranes) and 100% represents p97/p47wt (42.7% in cisternal membranes).

Competition experiments confirmed that the first p97-binding site actually binds to p97 (Fig. 1B). BSU peptide, which corresponds to the first p97-binding site in p47, stripped p47 from the p97-beads effectively. Hence, this first binding site alone is thought to have high binding affinity to p97 and to be a good target to produce a point-mutated p47 lacking p97-binding affinity.

3.2. Isolation of a point-mutated p47 lucking no p97-binding affinity

We next tried to produce p47 mutants which had a point mutation in either the first or second p97-binding site. The cDNA of p47(171–370), containing two p97-binding sites, was amplified by a PCR reaction with a limiting amount of dATP, and mutations were randomly induced in p47(171–370). The resulting p47(171–370) fragments were expressed in yeast cells and their p97-binding affinities were tested using a yeast two hybrid system. We screened $\sim 1 \times 10^3$ colonies and successfully obtained three mutants showing low p97-binding affinity; F253S, L339H and L355P.

We then expressed full-length p47 proteins with each mutation in *E. coli*. As a result, we found that p47(F253S) became soluble, but p47(L339H) and p47(L355P) became insoluble. The Phe-253 in p47 is conserved from mammals to yeast (Fig. 2A) and also exists in the first p97-binding site (244–270). Hence, we focused on the F253S mutant. Fig. 3B shows binding of p47wt and p47(F253S) to GST-tagged p97. p47wt bound to p97 efficiently (lane 1), while p47(F253S) showed no binding to p97 (lane 2). Since Beuron et al. predicted nucleotide-dependent conformational changes in p97 and p47 [17], we tested the effect of nucleotides on the binding of p47(F253S) to p97 and found that the mutant did not bind to p97 even in the presence of ATPγS or ADP (data not shown).

We next aimed to clarify that the effect of the F253S mutation in full-length p47 is mediated by the loss of p97-binding in the first binding site of p47, and hence carried out binding experiments using GST-tagged p47(244–270) which possessed only the first binding site (Fig. 2C). The F253S-mutated p47(244–270) fragment never bound to p97 (lane2). Therefore, the F253S mutation abolished the binding ability of the first p97-binding site, leading to lack of binding of full-length p47(F253S) to p97. Phe-253 is in a highly unstructured region of p47 [18] which could become structured upon binding to p97. The F253S mutation could alter the nature of the unstructured linker region, affecting the conformational changes. Also, the additional negative charges on the Ser could alter the local conformation, masking the interaction site with p97.

3.3. Complex formation between p97 and p47 is essential for p97/p47/VCIP135-mediated Golgi membrane fusion

In the p97/p47/VCIP135 pathway, p97 has two candidate binding partners, p47 and VCIP135, and it remains unclear whether complex formation between p97 and these molecules is really necessary for p97-mediated membrane fusion. Using p47(F253S) and BSU peptide, we tested this in an in vitro membrane fusion assay.

Fig. 3A shows the effects of p47 fragments on p97 ATPase activity. p47(171–270) fragment, which containing the first p97-binding site, inhibited p97 ATPase activity, but a smaller fragment containing the first p97-binding site, BSU peptide, did not. This suggests that BSU peptide is a good tool for inhibiting the binding of the first binding site in p47 to p97, without affecting p97 ATPase function. Fig. 3B demonstrates that the amount of p47(F253S) binding to Golgi membranes was almost the same as that of p47wt, which reveals that the binding ability of p47(F253S) to Golgi membranes is not changed. We also confirmed that the F253S mutation had no effect on p47 intracellular localization (data not shown).

Next, the effect of BSU peptide addition and the function of p47(F253S) in p97-mediated membrane fusion were investigated using an in vitro Golgi reassembly assay, as shown in Fig. 3C. The addition of BSU peptide decreased cisternal regrowth up to ~30% of the positive control. When p47(F253S) was added together with p97 to mitotic Golgi fragments, no cisternal regrowth was observed. Considering these results, the formation of the p97/p47 complex is thought to be required for p97/p47/VCIP135-mediated Golgi membrane fusion, and the interaction between p97 and the first binding site in p47 is especially important for the membrane fusion event.

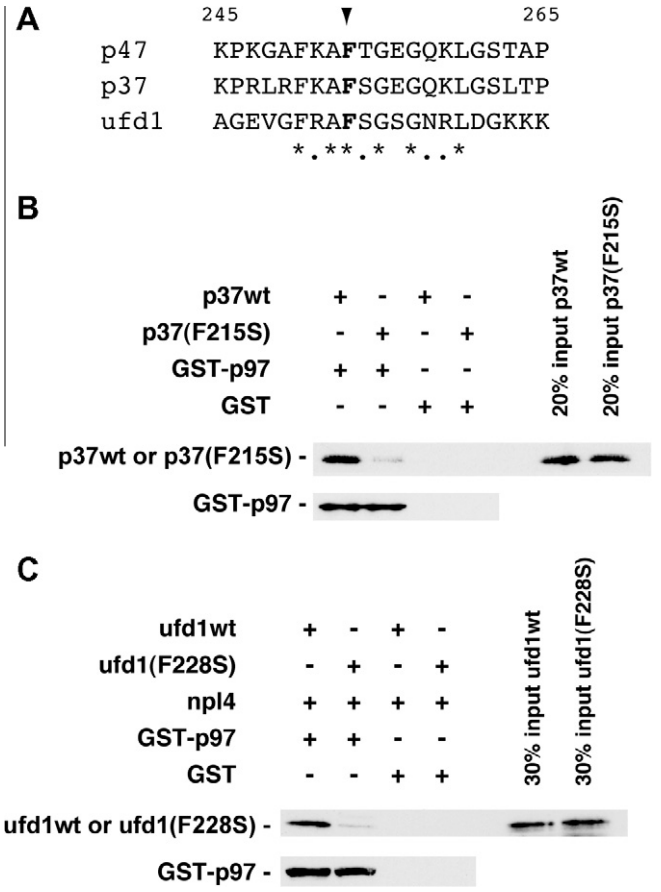

Fig. 4. Point-mutated p37 and ufd1. (A) Amino acid sequence alignment of p47, p37 and ufd1. Identical residues between homologues are marked with asterisks. The arrowhead indicates Phe-253 in p47. (B and C) GST-tagged p97 (0.8 μg) was immobilized to glutathione-beads. The beads were incubated with the indicated His-tagged proteins (p37, 0.04 μg; Ufd1, 0.04 μg; Npl4, 0.08 μg) in a buffer containing 0.15 M KCl and 0.1% Triton X-100 on ice. The blots were probed with antibodies to p97 and His-tag.

3.4. Point-mutated p37 and ufd1

As shown in Fig. 4A, sequence alignment of p47, p37 and Ufd1 reveals a short homologous stretch and Phe-253 in p47 is conserved in p37 and Ufd1. We therefore produced point-mutated p37 and Ufd1, p37(F215S) and Ufd1(F228S), corresponding to the F253S mutant of p47. Fig. 4B and C shows their binding to p97. As expected, neither p37(F215S) nor Ufd1(F228S) bound to p97. These results also confirm the importance of Phe-253 in p47 for its binding to p97. p37(F215S) and Ufd1(F228S) are thought to be dominant negative mutants lacking p97-binding affinity, which will hence be good tools for the analysis of their function.

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