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Phosphorylation of p37 is important for Golgi disassembly at mitosis

Yayoi Kaneko [a,b], Kaori Tamura [a], Go Totsukawa [a,b], Hisao Kondo [a,*]

[a] Department of Molecular Cell Biology, Graduate School of Medical Sciences, Kyushu University, Fukuoka 812-8582, Japan
[b] Mitsubishi Kagaku Institute of Life Sciences, Tokyo 194-8511, Japan

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ABSTRACT

In mammals, the Golgi apparatus is disassembled at early mitosis and reassembled at the end of mitosis. For Golgi disassembly, membrane fusion needs to be blocked. Golgi biogenesis requires two distinct p97ATPase-mediated membrane fusion, the p97/p47 and p97/p37 pathways. We previously reported that p47 phosphorylation on Serine-140 by Cdc2 results in mitotic inhibition of the p97/p47 pathway [11]. In this study, we demonstrate that p37 is phosphorylated on Serine-56 and Threonine-59 by Cdc2 at mitosis, and this phosphorylated p37 does not bind to Golgi membranes. Using an *in vitro* Golgi reassembly assay, we show that mutated p37(S56D, T59D), which mimics mitotic phosphorylation, does not cause any cisternal regrowth, indicating that p37 phosphorylation inhibits the p97/p37 pathway. Our results demonstrate that p37 phosphorylation on Serine-56 and Threonine-59 is important for Golgi disassembly at mitosis.

1. Introduction

The Golgi apparatus occupies a central position in the classical secretory pathway, where it receives the entire output of *de novo* synthesized proteins from the ER, and functions to distill, post-translationally process, and sort cargo to their ultimate destinations [1]. For Golgi inheritance at mitosis, there are several strategies: *de novo* formation, fission, and disassembly–reassembly [2,3]. In animal cells, the strategy of disassembly–reassembly is utilized [4]. During mitosis, the Golgi apparatus is fragmented into thousands of vesicles and short tubules that are dispersed throughout the cytoplasm. Some of them might be absorbed into ER [5]. At telophase, the Golgi apparatus is rapidly reassembled from the fragments within each daughter cell [6]. Golgi disassembly–reassembly requires the blocking of membrane fusion at early mitosis and its unblocking at late mitosis [4].

Experiments using an *in vitro* Golgi reassembly assay, which mimics the reassembly of Golgi stacks at the end of mitosis [7], showed that reassembly from membrane fragments requires at least two ATPases; *N*-ethylmaleimide-sensitive factor (NSF)[1] and p97ATPase [8]. For the NSF pathway, the tethering of p115-GM130 is disrupted by the mitotic phosphorylation of GM130, resulting in the inhibition of NSF-mediated fusion [9].

p97ATPase 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, and p37 is required for the maintenance of organelles during interphase as well as for their reassembly during mitosis [10–12]. We previously reported the mechanism of mitotic inhibition of p97/p47-mediated Golgi membrane fusion [11]. p47 is phosphorylated on Serine-140 by Cdc2 at mitosis. This phosphorylated p47 is unable to bind to Golgi membranes, resulting in mitotic inhibition of the p97/p47 pathway.

In this paper, we clarified the mechanism of mitotic inhibition of the other p97 pathway, the p97/p37 pathway. We show that p37 is phosphorylated on Serine-56 and Threonine-59 by Cdc2 at mitosis. Phosphorylation disables p37 from binding to Golgi membranes, and consequently blocks p97/p37-mediated Golgi membrane fusion at mitosis.

2. Materials and methods

2.1. Proteins, antibodies and reagents

Recombinant p97 and p37 were prepared as previously described [13,12]. Point mutations were directly introduced into the p37 cDNA in pQE30 by PCR reactions, using the Quik-change mutagenesis kit (Stratagene). All clones were verified by DNA sequencing.

Polyclonal antibodies to p37 and p97 were prepared as described [12,11]. Monoclonal antibodies to p97, His-tag and

* Corresponding author. Address: Department of Molecular Cell Biology, Graduate School of Medical Sciences, Kyushu University, 3-1-1 Maidashi, Fukuoka 812-8582, Japan. Fax: +81 92 642 6175.
E-mail address: hk228@med.kyushu-u.ac.jp (H. Kondo).

[1] *Abbreviations used:* NSF, *N*-ethylmaleimide-sensitive factor; His, six-histidine tag; GST, glutathione S-transferase

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GM130 were purchased from Progen, Qiagen and BD Transduction, respectively.

The following reagents were purchased from Calbiochem; staurosporine, olomoucine, PD98059, SB203580, KT5720, calphostin C, microcystin-LR. Cdc2 kinase (p34cdc2/cyclin B) was from New England Biolabs.

2.2. In vivo metabolic ^{32}P-labeling

For enrichment of mitotic Hela cells, aphidicolin (2.5 μg/ml) was added to the culture medium for 14 h. The cells were then washed with fresh medium, released from the S phase-block for 2 h, and labeled with ^{32}P-orthophosphate (200 μCi/ml) for another 4 h at 37 °C. Mitotic cells were flushed from the dish, washed with PBS, and extracted with buffer (50 mM Tris, 1 mM EDTA, 1% SDS, 6 M Urea, pH 7.4). The lysate was cleared by centrifugation and used for the denatured immunoprecipitation with anti-p37 antibodies.

2.3. In vitro phosphorylation

p37 or its mutant was incubated in Buffer A (50 mM Tris, 50 mM KOAc, 10 mM MgOAc, 20 mM β-glycerophosphate, 0.2 mM DTT, 40 μM ATP, 30 μCi/μl [γ-^{32}P]ATP, pH 7.4) with mitotic cytosol or purified kinase for 30 min at 30 °C, followed by denatured immunoprecipitation. The reactions were terminated by adding an equal volume of buffer (100 mM Tris, 2 mM EDTA, 2% SDS, pH 7.4) and boiled for 4 min. After adding 20 volumes of buffer (50 mM Tris, 0.15 M KCl, 0.5% Tween-20, 1 mM EDTA, 1 mM EGTA, 10 mM NaF, 40 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM DTT, pH 7.4), p37 or its mutant was immunoprecipitated with antibodies to a His-tag and protein G-beads, followed by SDS–PAGE and autoradiography.

For the binding experiments of p37 to salt-washed Golgi membranes or GST-p97, 1 mM ATP was added to Buffer A instead of 40 μM ATP and 30 μCi/μl [γ-^{32}P]ATP. After incubation, the reactions were supplemented with microcystin-LR (10 μM) and staurosporine (25 μM), followed by the binding experiments using Golgi membranes or GST-p97.

2.4. Binding experiments and immunoprecipitation

Golgi membranes were purified from rat liver as described previously [14]. KCl-washed Golgi membranes (1 M) and mitotic Golgi fragments were prepared as described previously [13]. p37 or its mutant was incubated with salt-washed Golgi membranes in buffer (0.1 M KCl, 20 mM Tris, 1 mM $MgCl_2$, 1 mM ATP, 0.4 g/l BSA, 0.2 M sucrose, pH 7.4) for 1 h on ice, and then the membranes were recovered by centrifugation.

For the binding experiments with GST-p97 and the immunoprecipitation experiments with cytosol, the reactions were performed in buffer (0.15 M KCl, 20 mM Hepes, 1 mM $MgCl_2$, 1 mM DTT, 0.1% Triton X-100, 5% glycerol, pH 7.4).

2.5. In vitro Golgi reassembly assay

The *in vitro* Golgi reassembly assays were performed as reported previously [9,15].

3. Results and discussion

3.1. p37 is mitotically phosphorylated

To investigate whether there was mitotic phosphorylation of p37, Hela cells were synchronized and incubated in medium containing ^{32}P-orthophosphate. Mitotic cells were collected by mechanical shake-off and used for the preparation of ^{32}P-labeled mitotic cell lysate. Hoechst DNA staining showed that more than 95% of cells were mitotic (data not shown). Non-synchronized cells were used for the preparation of interphase cell lysate. p37 was isolated from the lysate by denatured immunoprecipitation. Autoradiography revealed that p37 was strongly phosphorylated at mitosis (Fig. 1A, bottom panel).

We next aimed to determine the responsible kinase. At first, His-p37 was incubated with mitotic cytosol in the presence of [γ-^{32}P]ATP. As presented in Fig. 1B, p37 was strongly phosphorylated in mitotic cytosol (lane 2). We next investigated the effect of several protein kinase inhibitors on the mitotic phosphorylation of p37 (Fig. 1B, lanes 3–8). Staurosporine (broad serine/threonine kinase inhibitor) and olomoucine (Cdc2 inhibitor) inhibited p37 phosphorylation (lanes 3 and 4). On the other hand, neither PD98059 (MEK inhibitor), SB203580 (p38 MAP kinase inhibitor), KT5720 (protein kinase A inhibitor) nor calphostin C (protein kinase C inhibitor) had any effect on p37 phosphorylation (lanes 5–8). Hence, Cdc2 was thought to be a candidate kinase. To confirm this, we investigated whether the purified Cdc2 complex phosphorylated p37 in the absence of mitotic cytosol, as shown in Fig. 1C. Cdc2 strongly phosphorylated p37 (right lane). Therefore, Cdc2 was confirmed as the kinase responsible for p37 phosphorylation.

We tried to determine the phosphorylation sites in p37. Fig. 1D shows that p37(1–101) was phosphorylated by mitotic cytosol (middle lane), while p37(102–331) was not (right lane). The consensus motif for phosphorylation by Cdc2 is Ser/Thr-Pro-X-Arg/Lys, with Pro at the +1 position being absolutely critical, and a basic residue at the +3 position preferred but not essential for kinase recognition [16]. p37(1–101) has two Thr and one Ser residue with Pro at the +1 position: T50, S56 and T59. p37 was mutated at each of these sites and tested for its phosphorylation. Both S56A and T59A mutation reduced p37 phosphorylation by mitotic cytosol (lanes 3 and 4), while the T50A mutation did not show any effect (lane 2). When both S56A and T59A mutations were induced into p37 (p37(S56A, T59A)), phosphorylation of p37 by mitotic cytosol was completely inhibited (lane 5). Similar results were obtained by using purified Cdc2 instead of mitotic cytosol (data not shown). Taken together, we conclude that p37 is mitotically phosphorylated on Serine-56 and Threonine-59 by Cdc2.

3.2. Phosphorylated p37 maintains its binding to p97

Determination of the phosphorylation of p37 at mitosis led to the question of what effect this phosphorylation has on the function of p37. p37 forms a tight complex with p97, which is essential for *in vitro* Golgi reassembly [12]. We therefore next investigated the effect of phosphorylation on the binding of p37 to p97.

To test whether p97 binds phosphorylated p37, p37 was phosphorylated by Cdc2 in the presence of [γ-^{32}P]ATP, and then incubated with GST-p97 (Fig. 2A). GST-p97 and its binding proteins were recovered with GSH-beads, and phosphorylated p37 was detected by autoradiography. Phosphorylated p37 was co-precipitated with GST-p97 (middle lane), suggesting that phosphorylated p37 binds to p97.

Since p97 is known to be phosphorylated at mitosis [17], we also investigated whether p37 really forms a complex with p97 in mitotic cytosol. Fig. 2B shows immunoprecipitation experiments using anti-p37 antibodies. There was no difference in the amount of co-precipitated p97 between interphase and mitotic cytosol (top panel). Thus, all the data from the binding and immunoprecipitation experiments show that p37 phosphorylation has no effect on complex formation with p97.

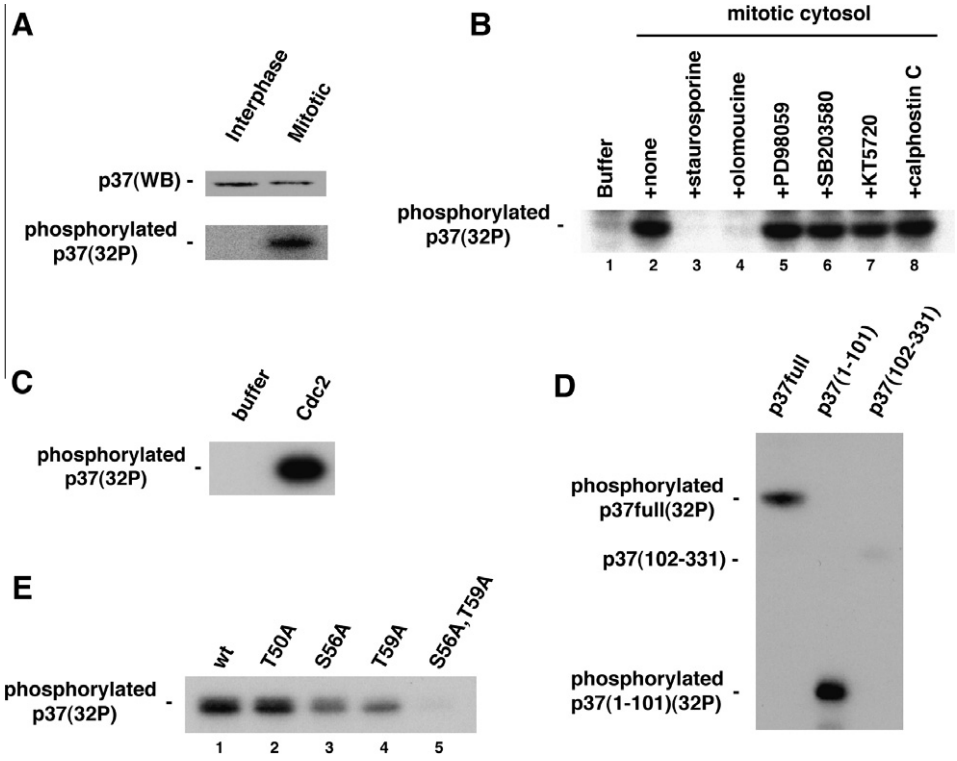

Fig. 1. p37 is mitotically phosphorylated by Cdc2. (A) Mitotic phosphorylation of p37 *in vivo*. p37 was immunoprecipitated from either ^{32}P-labeled interphase or mitotic cell lysates under a denatured condition. After fractionation by SDS–PAGE, p37 was detected by Western blotting with anti-p37 antibodies (upper panel) and ^{32}P-labeled p37 was detected by autoradiography (lower panel). (B) Effect of protein kinase inhibitors on p37 phosphorylation in mitotic cytosol. His-tagged p37 (40 μg/ml) was incubated for 1 h at 30 °C in the presence of mitotic cytosol (10 mg protein/ml) and [γ-^{32}P]ATP with the indicated protein kinase inhibitor as follows: 10 μM staurosporine, 1 mM olomoucine, 50 μM PD98059, 100 μM SB203580, 10 μM KT5720, 500 nM calphostin C. After incubation, His-p37 was immunoprecipitated with anti-His antibodies under a denatured condition and phospholabeled His-p37 was detected as in (A). (C) Direct phosphorylation of p37 by Cdc2. His-p37 (40 μg/ml) was incubated in the presence of [γ-^{32}P]ATP with buffer or recombinant Cdc2 complex (60 mU/ml). (D) Phosphorylation sites of p37 exist in its N-terminal region. Either His-p37full (40 μg/ml), His-p37(1–101) (15 μg/ml) or His-p37(102–331) (30 μg/ml) was incubated for 1 h at 30 °C in the presence of mitotic cytosol (10 mg protein/ml) and [γ-^{32}P]ATP. (E) Cdc2-phosphorylation sites in p37. Either p37wt or p37 mutant (40 μg/ml) was incubated in the presence of [γ-^{32}P]ATP with mitotic cytosol (10 mg protein/ml).

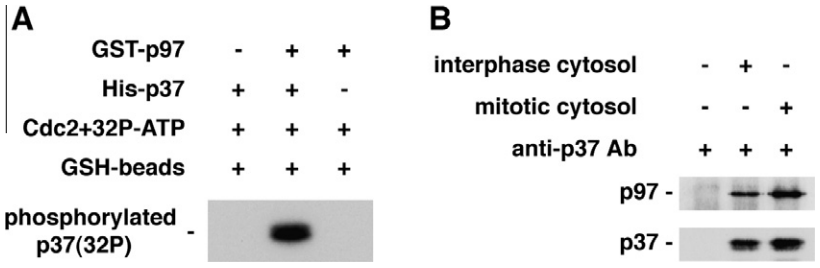

Fig. 2. p37 forms a complex with p97 in mitotic cytosol. (A) Phosphorylated p37 binds to p97. p37 (5 μg/ml) was incubated with [γ-^{32}P]ATP and Cdc2 (80 mU/ml) for 1 h at 30 °C, followed by the addition of staurosporine and microcystin-LR. The solution was incubated with GST-tagged p97 (20 μg/ml) and GSH-beads. Proteins bound to the beads were fractionated by SDS–PAGE. ^{32}P-labeled p37 was detected by autoradiography. (B) p37 forms a complex with p97 in mitotic cytosol as well as in interphase cytosol. Either interphase or mitotic cytosol was incubated with anti-p37 antibodies. The immunoprecipitates were fractionated by SDS–PAGE, and blots were probed with antibodies to p37 and p97.

3.3. Phosphorylation of Serine-56 and Threonine-59 in p37 inhibits its binding to Golgi membranes

It has been reported that p37 mediates binding of the p97/p37 complex to Golgi membranes [12]. We therefore investigated the effect of p37 phosphorylation on its binding to Golgi membranes. We first tested whether phosphorylated p37 binds to Golgi membranes. p37 was phosphorylated by incubation with Cdc2 and then its phosphorylated state was 'frozen' by addition of staurosporine and microcystin-LR. The phosphorylated p37 was then used for binding experiments with salt-washed Golgi membranes. As presented in Fig. 3A, Cdc2-mediated p37 phosphorylation inhibited its binding to Golgi membranes (upper panel, lane 3). The inhibition was rescued by olomoucine, an inhibitor of Cdc2 (upper panel, lane 4). This inhibition and rescue of Golgi binding was also observed when p37 alone was added instead of a p97/p37 complex (data not shown).

Next, a p37 mutant, p37(S56A, T59A), which cannot be phosphorylated at Serine-56 and Threonine-59, was used for the binding experiments (Fig. 3B). p37(S56A, T59A) bound to Golgi membranes (Fig. 3B, upper panel, lane 2) and Cdc2 had no effect on this binding (lane 3). We finally tested another p37 mutant, p37(S56D, T59D), which mimics the phosphorylation of Serine-56 and Threonine-59, for the binding experiments (Fig. 3C). Much

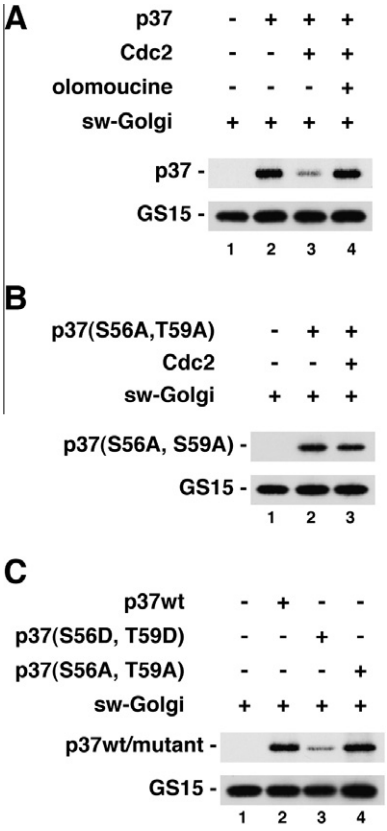

Fig. 3. Phosphorylated p37 does not bind to Golgi membranes. (A) p37 (1.5 μg) was incubated with Cdc2 (30 U) in the absence or presence of olomoucine (1 mM) for 1 h at 30 °C, followed by the addition of staurosporine and microcystin-LR. The solution was then incubated with p97 (4 μg) and 1 M KCl-washed Golgi membranes (30 μg), and the membranes were isolated from unbound p37 by centrifugation, followed by Western blotting. Blots were probed by monoclonal antibodies to the His-tag on p37 and polyclonal antibodies to GS15. (B) p37(S56A, T59A) was used instead of p37wt in the experiments presented in (A). Blots were probed by monoclonal antibodies to the His-tag on p37(S56A, T59A) and polyclonal antibodies to GS15. (C) Binding of p37 mutants to Golgi membranes. 1 M KCl-washed Golgi membranes (30 μg) were incubated with either p37wt or p37 mutant (1.5 μg) in the presence of p97 (4 μg). Blots were probed by monoclonal antibodies to the His-tag on p37s and polyclonal antibodies to GS15.

less p37(S56D, T59D) bound to Golgi membranes (Fig. 3C, upper panel, lane 3) compared with p37wt (lane 2) and p37(S56A, T59A) (lane 4). In summary, our biochemical data show that phosphorylation of Serine-56 and Threonine-59 in p37 inhibits its binding to Golgi membranes.

Serine-56 and Threonine-59 are in a highly unstructured region (X. Yuan and S. Matthews, Imperial College, London, UK; personal communication) which could become structured upon binding to SNAREs. Phosphorylation could alter the nature of the unstructured loop region, affecting the conformational changes. Also, the additional negative charges on phosphorylated Serine-56 and Threonine-59 could alter the local conformation of the structure, masking the interaction site of p37 with SNAREs.

3.4. Phosphorylation of Serine 56 and Threonine 59 in p37 inhibits p97/p37-mediated Golgi membrane fusion in vitro

We established that p37 is mitotically phosphorylated at Serine-56 and Threonine-59 and that phosphorylated p37 is dissociated from Golgi membranes. Next, we aimed to determine whether this phosphorylation of p37 has any effect on Golgi assembly *in vitro*. We tested the effect of p37(S56D, T59D), which mimics Serine-56 and Threonine-59 phosphorylation, on Golgi

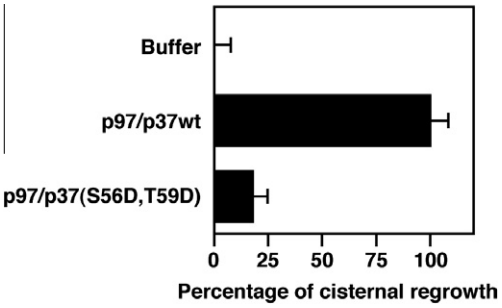

Fig. 4. The effects of p37 with mutated phosphorylation sites on Golgi reassembly *in vitro*. Mitotic Golgi membranes were incubated with the indicated components at 37 °C for 60 min: p97/p37wt (60 μg p97/ml), p97/p37(S56D, T59D) (60 μg p97/ml). Results are presented as percentage of cisternal regrowth ± SD ($n = 4$): 0% represents the buffer (25.6% in cisternal membranes) and 100% represents p97/p37wt (38.9% in cisternal membranes).

reassembly. Mitotic Golgi fragments were incubated with p97/p37wt or p97/p37(S56D, T59D) for the *in vitro* Golgi reassembly assay. Fig. 4 shows that p97/p37(S56D, T59D) had a very small effect on cisternal regrowth compared with p97/p37wt, suggesting that the phosphorylation of Serine-56 and Threonine-59 in p37 inhibits p97/p37-mediated Golgi membrane fusion. Hence, p37 phosphorylation is thought to be important for Golgi disassembly at mitosis.

Mitotic inhibition of the NSF pathway for Golgi disassembly is achieved by blocking the tethering of p115-GM130 [18], and its inhibition is rescued by dephosphorylation of GM130 at telophase [19]. Since p97/p37-mediated Golgi membrane fusion also requires p115-GM130 tethering [12], the p97/p37 pathway is thought to utilize phosphorylation–dephosphorylation of GM130 for its mitotic control in the same way as the NSF pathway. Together with our finding of mitotic p37 phosphorylation, the p97/p37-mediated Golgi disassembly at mitosis is achieved in two distinct ways, by p37 and GM130 phosphorylation, and this binary system might enable very tight and precise control of Golgi disassembly–reassembly at mitosis.

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