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Mitotic phosphorylation of VCIP135 blocks p97ATPase-mediated Golgi membrane fusion

Go Totsukawa, Ayaka Matsuo, Ayano Kubota, Yuya Taguchi, Hisao Kondo *

Department of Molecular Cell Biology, Graduate School of Medical Sciences, Kyushu University, Fukuoka 812-8582, Japan

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ABSTRACT

In mammals, the Golgi apparatus is disassembled 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 and p37 phosphorylation on Serine-56 and Threonine-59 result in mitotic inhibition of the p97/p47 and the p97/p37 pathways, respectively [11,14]. In this study, we show another mechanism of mitotic inhibition of p97-mediated Golgi membrane fusion. We clarified that VCIP135, an essential factor in both p97 membrane fusion pathways, is phosphorylated on Threonine-760 and Serine-767 by Cdc2 at mitosis and that this phosphorylated VCIP135 does not bind to p97. An *in vitro* Golgi reassembly assay revealed that VCIP135(T760E, S767E), which mimics mitotic phosphorylation, caused no cisternal regrowth. Our results indicate that the phosphorylation of VCIP135 on Threonine-760 and Serine-767 inhibits p97-mediated Golgi membrane fusion 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 these are thought to be absorbed into the 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) and p97ATPase [8]. In 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]. Both p97 membrane fusion pathways require VCIP135(VCP(p97)/p47 Complex-Interacting-Protein, p135) [13]. We previously reported some of the mechanisms of mitotic inhibition of p97-mediated Golgi membrane fusion [11,14]. At mitosis, Cdc2 kinase phosphorylates p47 on Serine-140 and p37 on Serine-56 and Threonine-59, respectively. The phosphorylated p47 and p37 are unable to bind to Golgi membranes, resulting in mitotic inhibition of the p97/p47 and the p97/p37 pathways, respectively.

In this study, we identified another mechanism of mitotic inhibition of both p97 pathways. We show that VCIP135 is phosphorylated on Threonine-760 and Serine-767 by Cdc2 at mitosis. Phosphorylation disables VCIP135 from binding to p97, and consequently blocks p97-mediated Golgi membrane fusion at mitosis.

2. Materials and methods

2.1. Proteins, antibodies and reagents

Recombinant VCIP135, p97, WAC, p47 and p37 were prepared as previously described [10,12,15]. Point mutations were directly introduced into the VCIP135 cDNA in pTrcHis by PCR reactions,

Abbreviations: VCP135, VCP(p97)/p47 complex-interacting-protein p135; WAC, WW domain-containing adaptor with coiled-coil; NSF, *N*-ethylmaleimide-sensitive factor; His, six-histidine tag; GST, glutathione S-transferase.

* 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).

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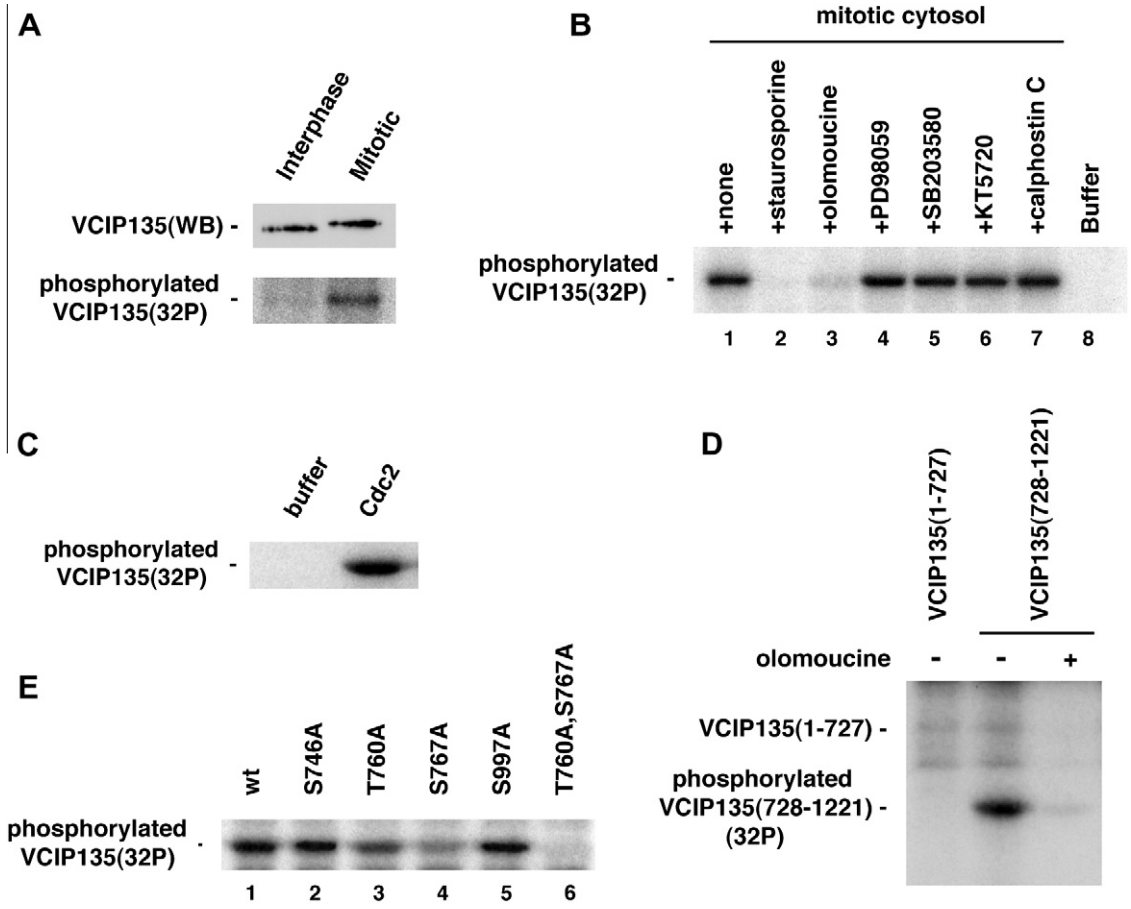

Fig. 1. VCIP135 is mitotically phosphorylated by Cdc2. (A) Mitotic phosphorylation of VCIP135 *in vivo*. VCIP135 was immunoprecipitated from either ^{32}P-labeled interphase or mitotic Hela cell lysates under a denatured condition. After fractionation by SDS–PAGE, VCIP135 was detected by Western blotting with an anti-VCIP135 antibody (upper panel) and ^{32}P-labeled VCIP135 was detected by autoradiography (lower panel). (B) Effect of protein kinase inhibitors on VCIP135 phosphorylation in mitotic cytosol. His-tagged VCIP135 (50 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, or 500 nM calphostin C. After incubation, His-VCIP135 was immunoprecipitated with an anti-His antibody under a denatured condition and phospholabeled His-VCIP135 was detected as in (A). (C) Direct phosphorylation of VCIP135 by Cdc2. His-VCIP135 (50 μg/ml) was incubated in the presence of [γ-^{32}P]ATP with buffer or recombinant Cdc2 complex (1000 Units/ml). (D) Phosphorylation sites of VCIP135 exist in its C-terminal region. Either His-VCIP135(1–727) (30 μg/ml) or His-VCIP135(728–1221) (20 μg/ml) was incubated for 1 h at 30 °C with mitotic cytosol (10 mg protein/ml) and [γ-^{32}P]ATP. The incubation of His-VCIP135(728–1221) was performed in the absence or presence of olomoucine (1 mM). (E) Cdc2-phosphorylation sites in VCIP135. Either VCIP135wt or VCIP135 mutant (50 μg/ml) was incubated in the presence of [γ-^{32}P]ATP with mitotic cytosol (10 mg protein/ml).

using the Quikchange mutagenesis kit (Stratagene). All clones were verified by DNA sequencing.

A polyclonal antibody to VCIP135 was prepared as described [11]. Monoclonal antibodies to p97, His-tag, GST and ubiquitin were purchased from Progen, Qiagen, Covance and Santa Cruz, respectively.

The following reagents were purchased from Calbiochem; staurosporine, olomoucine, PD98059, SB203580, KT5720, calphostin C, and 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 (20 mM Hepes, 0.1 M KCl, 5 mM EDTA, 2 mM EGTA, 40 mM -glycerophosphate, 10 mM NaF, 1 mM sodium orthovanadate, 1 mM DTT, protease inhibitor cocktail, 0.5% Triton X-100, 10% glycerol, pH 7.4). The lysate was cleared by centrifugation and used for the denatured immunoprecipitation with an anti-VCIP135 antibody.

2.3. In vitro phosphorylation

VCIP135 or its mutant was incubated in Buffer A (50 mM Tris, 50 mM KOAc, 10 mM MgOAc, 20 mM -glycerophosphate, 0.2 mM DTT, 40 μM ATP, 30 μCi/μl [^{32}P-γ]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 NaCl, 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), VCIP135 or its mutant was immunoprecipitated with an antibody to a His-tag and protein G-beads, followed by SDS–PAGE and autoradiography.

2.4. Binding experiments and deubiquitinase assays

The binding experiments with GST-p97 and GST-WAC 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). The deubiquitinating activity of VCIP135 was assayed as reported previously [15].

2.5. Immunoprecipitation from cytosol

Either interphase or mitotic cytosol (~0.1 mg total protein) was mixed with a polyclonal anti-VCIP135 antibody in buffer (0.1 M KCl, 20 mM Hepes, 1 mM $MgCl_2$, 1 mM DTT, 0.1% Triton X-100, 5% glycerol, pH 7.4)). VCIP35 and its binding proteins were immunoprecipitated by protein A beads.

2.6. In vitro Golgi reassembly assay

The *in vitro* Golgi reassembly assays were performed as reported previously [16,13,12]. For the preparation of salt-washed membranes, KCl was added into the mixture to 1 M, incubated on ice for 30 min, and the membranes were recovered by centrifugation.

3. Results and discussion

3.1. VCIP135 is mitotically phosphorylated

To investigate whether there was mitotic phosphorylation of VCIP135, 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. Non-synchronized cells were used for the preparation of interphase cell lysate. VCIP135 was isolated from the lysate by denatured immunoprecipitation. Autoradiography revealed that VCIP135 was phosphorylated at mitosis (Fig. 1A, bottom panel).

We next aimed to determine the kinase responsible for this phosphorylation. At first, VCIP135 was incubated with mitotic cytosol in the presence of [γ-^{32}P]ATP. As presented in Fig. 1B, VCIP135 was strongly phosphorylated in mitotic cytosol (lane 1). We next investigated the effect of several protein kinase inhibitors on the mitotic phosphorylation of VCIP135 (Fig. 1B, lanes 2–7). Staurosporine (broad serine/threonine kinase inhibitor) and olomoucine (Cdc2 inhibitor) inhibited VCIP135 phosphorylation (lanes 2 and 3). 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 VCIP135 phosphorylation (lanes 4–7). Hence, Cdc2 was thought to be a candidate kinase. To confirm this, we investigated whether the purified Cdc2 complex phosphorylated VCIP135 in the absence of mitotic cytosol. As shown in Fig. 1C, Cdc2 indeed phosphorylated VCIP135 (right lane). Therefore, Cdc2 was confirmed as the kinase responsible for VCIP135 phosphorylation.

We next tried to determine the phosphorylation sites in VCIP135. Fig. 1D shows that VCIP135(728–1221) was phosphorylated by mitotic cytosol (middle lane), while VCIP135(1–727) was not (left lane). The phosphorylation of VCIP135(728–1221) was also suppressed by the Cdc2 inhibitor, olomoucine (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 [17]. VCIP135(728–1221) has one Thr and three Ser residues with Pro at the +1 position: S764, T760, S767 and S997. VCIP135 was therefore mutated at each of these sites and tested for its phosphorylation. Both the T760A and S767A mutation reduced VCIP135 phosphorylation by mitotic cytosol (Fig. 1E, lanes 3 and 4), while neither the S764A nor S997A mutation showed any effect (lanes 2 and 5). When both the T760A and S767A mutations were induced into VCIP135 (VCIP135(T760A, S767A)), phosphorylation of VCIP135 by mitotic cytosol was inhibited (lane 6). Similar results were obtained by using purified Cdc2 instead of mitotic cytosol (data not shown). Taken together, we conclude that VCIP135 is mitotically phosphorylated on Threonine-760 and Serine-767 by Cdc2.

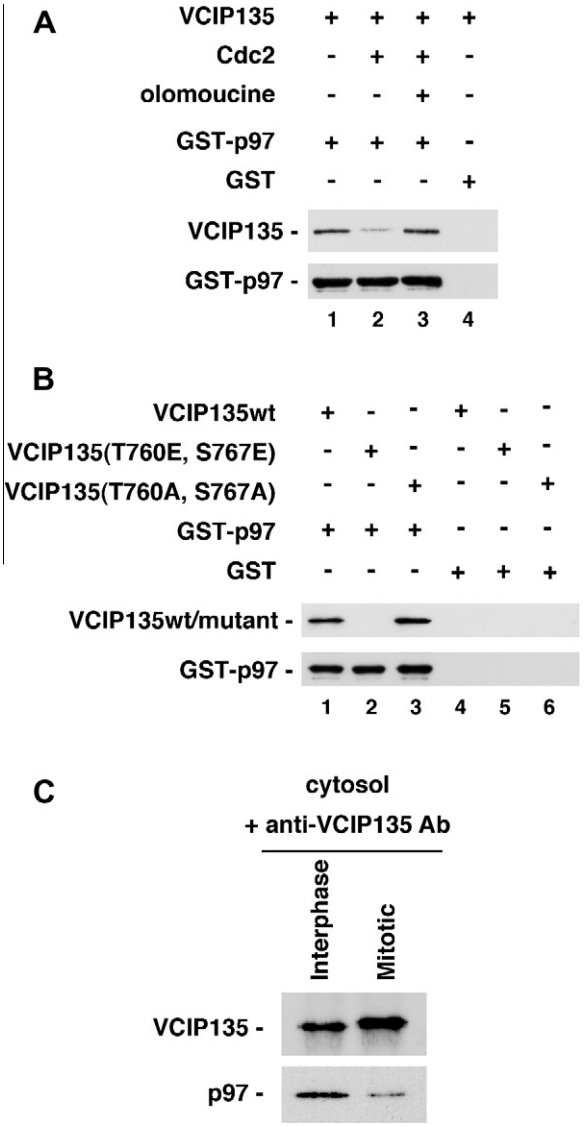

Fig. 2. Phosphorylated VCIP135 does not bind to p97. (A) His-VCIP135 (1.5 μg) was incubated with Cdc2 (6 Units) in the absence or presence of olomoucine (1 mM) for 1 h at 30 °C, followed by the addition of staurosporine and microcystin-LR. The solution was then incubated with GST- p97 (2 μg) and GSH-beads. Proteins bound to the beads were fractionated by SDS–PAGE. Blots were probed with monoclonal antibodies to p97 and the His-tag on VCIP135. (B) Binding of VCIP135 mutants to p97. GST-p97 (2 μg) was incubated with either VCIP135wt or VCIP135 mutant (0.5 μg). Blots were probed as in (A). (C) Either interphase or mitotic Hela cytosol was incubated with a polyclonal anti-VCIP135 antibody. The immunoprecipitates were fractionated by SDS–PAGE, followed by Western blotting with a polyclonal antibody to VCIP135 and a monoclonal antibody to p97.

3.2. Phosphorylation of Threonine-760 and Serine-767 in VCIP135 inhibits its binding to p97

Determination of the phosphorylation of VCIP135 at mitosis led to the question as to what effect this phosphorylation has on the function of VCIP135. VCIP135 shows binding affinities for p97

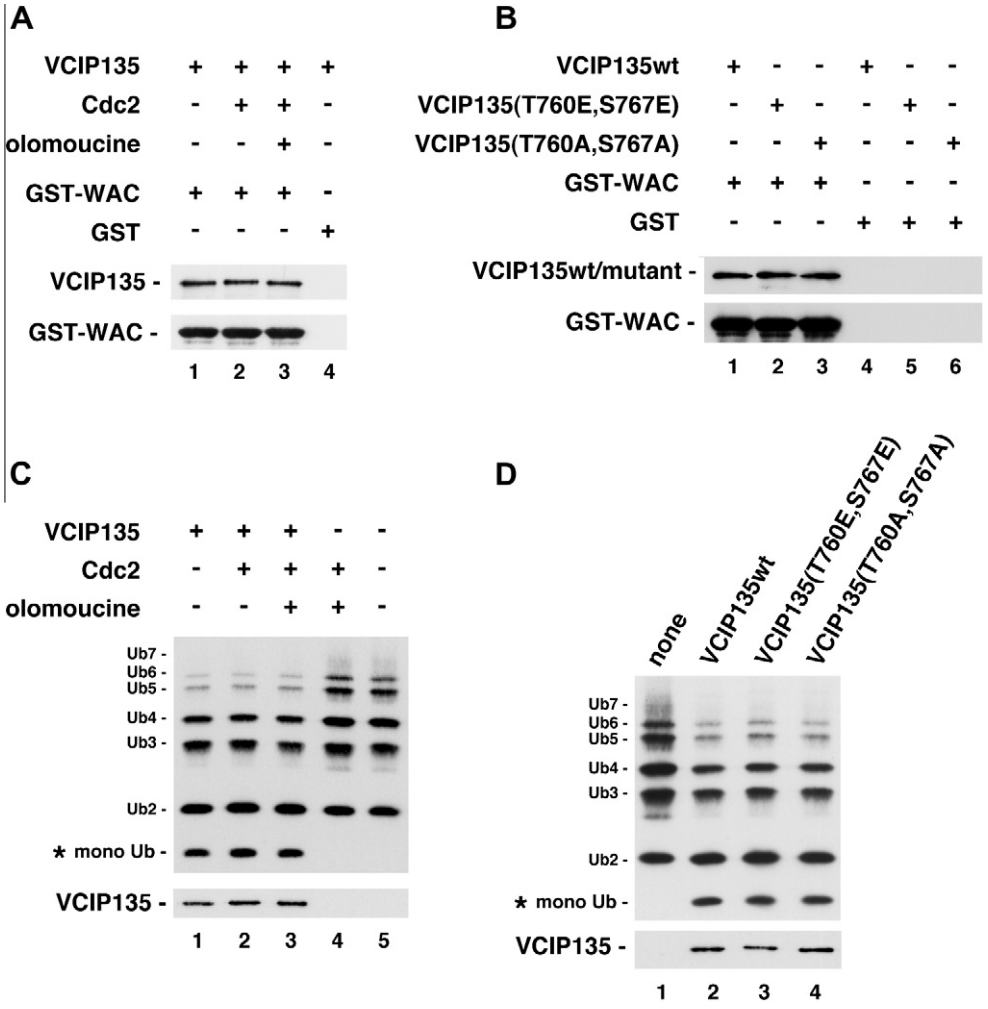

Fig. 3. Phosphorylated VCIP135 binds to WAC and still maintains a deubiquitinating activity. (A) His-VCIP135 (1.5 μg) was incubated with Cdc2 (6 Units) in the absence or presence of olomoucine (1 mM) for 1 h at 30 °C, followed by the addition of staurosporine and microcystin-LR. The solution was then incubated with GST-WAC (4 μg) and GSH-beads. Proteins bound to the beads were fractionated by SDS–PAGE. Blots were probed with a monoclonal antibody to the His-tag on VCIP135 and a polyclonal antibody to WAC. (B) Binding of VCIP135 mutants to WAC. GST-WAC (4 μg) was incubated with either VCIP135wt or VCIP135 mutant (2 μg). Blots were probed as in (A). (C) Phosphorylated VCIP135 still maintains a deubiquitinating activity. His-VCIP135 (1.5 μg) was incubated with Cdc2 (6 Units) 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 incubated with a mixture of *in vitro*-synthesized ubiquitin chains (Ub2–7, 10 μg/ml) and then analyzed by Western blotting with antibodies to ubiquitin and the His-tag on VCIP135. (D) VCIP135 mutants still maintain a deubiquitinating activity. A mixture of *in vitro*-synthesized ubiquitin chains (Ub2–7, 10 μg/ml) was incubated alone, with His-VCIP135wt (40 μg/ml), or with His-VCIP135mutant (40 μg/ml), and then analyzed as in (C).

and WAC (WW domain-containing adaptor with coiled-coil) [12,15]. We therefore investigated the effect of phosphorylation on the binding of VCIP135 to p97 or WAC.

We first tested whether phosphorylated VCIP135 binds to p97. VCIP135 was phosphorylated by incubation with Cdc2, and then its phosphorylated state was 'frozen' by addition of staurosporine and microcystin-LR. The phosphorylated VCIP135 was then used for binding experiments with GST-p97. As presented in Fig. 2A, Cdc2-mediated VCIP135 phosphorylation inhibited its binding to GST-p97 (upper panel, lane 2). The inhibition was rescued by olomoucine, an inhibitor of Cdc2 (upper panel, lane 3). Next, a VCIP135 mutant, VCIP135(T760E, S767E), which mimics the phosphorylation of Threonine-760 and Serine-767, was tested for its binding to p97 (Fig. 2B). Much less VCIP135(T760E, S767E) bound to GST-p97 (Fig. 2B, upper panel, lane 2) compared with VCIP135wt (lane 1). VCIP135(T760A, S767A), which is a mutant in which the same amino acid residues are mutated into Alanine, did not demonstrated decreased binding affinity to GST-p97 (lane 3). We also investigated whether the complex of VCIP135 and p97 existed in mitotic cytosol. As shown in Fig. 2C, very little p97 was co-immunoprecipitated by a polyclonal anti-VCIP135 antibody from mitotic cytosol (lower panel, right lane). All these results indicate that phosphorylation of Threonine-760 and Serine-767 in VCIP135 inhibits its binding to p97.

We next tested the binding of phosphorylated VCIP135 to WAC, as presented in Fig. 3A. Cdc2-phosphorylated VCIP135 still bound to GST-WAC (upper panel, lane 2), suggesting that the phosphorylation of VCIP135 has no effect on its binding to WAC. This was confirmed by the finding that VCIP135(T760E, S767E) showed almost the same binding affinity to GST-WAC as VCIP135 wild type (Fig. 3B, upper panel, lane 2).

The deubiquitinating enzyme activity of phosphorylated VCIP135 was also assayed. A mixture of ubiquitin chains (Ub2–7) was used as a substrate. VCIP135 phosphorylated by Cdc2 generated almost the same amount of mono ubiquitin after the reaction as non-phosphorylated VCIP135 (Fig. 3C, top panel, lane 2). The deubiquitinating activity of VCIP135(T760E, S767E) was also the same as that of the wild type (Fig. 3D, top panel, lane 3). Therefore, the phosphrylation of VCIP135 appears to have no effect on its deubiquitinating activity.

In summary, our biochemical data show that phosphorylation of Threonine-760 and Serine-767 in VCIP135 inhibits its binding

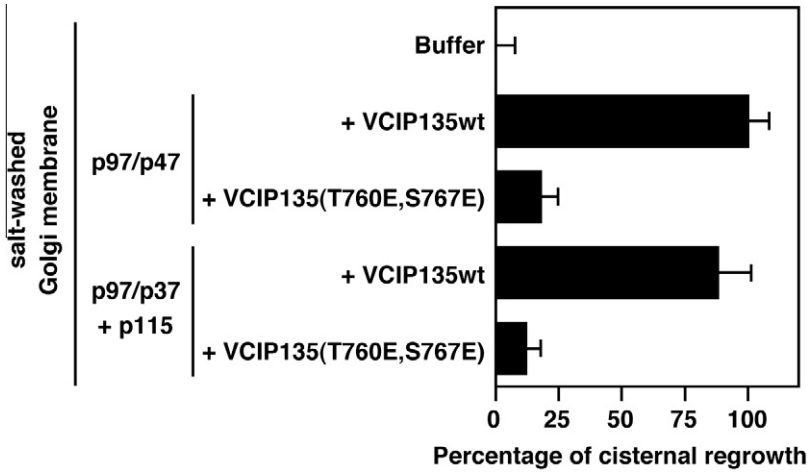

Fig. 4. The effects of VCIP135 with mutated phosphorylation sites on Golgi reassembly *in vitro*. VCIP135(T760E, S767E), which mimics the phosphorylation of Threonine-760 and Serine-767, did not function in p97-mediated Golgi reassembly. 1 M KCl-washed Golgi fragments were incubated with several components or their combinations; p97 (60 μg/ml), p47 (27 μg/ml), p37 (20 μg/ml), p115 (40 μg/ml), VCIP135wt (20 μg/ml), VCIP135(T760E, S767E) (20 μg/ml). The percentage of membrane in cisterna was determined. Mean +SD (n = 5–6); 0% represents the buffer (27.8% in cisternal membranes), 100% represents +p97/p47+VCIP135wt (50.7% in cisternal membranes).

to p97, but does not affect its binding to WAC nor its deubiquitinating activity.

3.3. Phosphorylation of Threonine-760 and Serine-767 in VCIP135 inhibits p97-mediated Golgi assembly in vitro

We established that VCIP135 is mitotically phosphorylated at Threonine-760 and Serine-767 and that the phosphorylated VCIP135 cannot bind to p97. To clarify the significance of this mitotic phosphorylation of VCIP135 in Golgi biogenesis, we performed the *in vitro* Golgi reassembly assay (Fig. 4) [12]. We tested VCIP135(T760E, S767E), which mimics Threonine-760 and Serine-767 phosphorylation, instead of phosphorylated VCIP135. Golgi membranes were washed with 1 M KCl to remove membrane-bound VCIP135 before being used for the assays.

It is known that there are two distinct p97 pathways for Golgi reassembly, the p97/p47 and p97/p37 pathways, and both pathways require VCIP135 [12]. We first investigated the function of VCIP135(T760E, S767E) in p97/p47-mediated Golgi assembly. When the salt-washed membranes were incubated with p97/p47 and VCIP135wt, cisternal growth was observed. However, when VCIP135(T760E, S767E) was added together with p97/p47, there was no cisternal growth. As to the other p97 pathway, p97/p37-mediated Golgi assembly, VCIP135(T760E, S767E) also did not cause cisternal growth in the presence of p97/p37 and p115. Taken together, these results demonstrate that the phosphorylation of Threonine-760 and Serine-767 in VCIP135 inhibits both p97-mediated Golgi reassembly pathways, indicating that it is important for Golgi disassembly at mitosis.

Mitotic inhibition of membrane fusion is essential for Golgi disassembly at mitosis. In the NSF pathway, the tethering of p115-GM130 is blocked by the phosphorylation of GM130 at mitosis [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. We also reported that p37 was phosphorylated on Serine-56 and Threonine-59 by Cdc2 at mitosis and that this phosphorylated p37 did not bind to Golgi membranes [14]. Hence, the p97/p37 pathway is inhibited at mitosis in two distinct ways, by the phosphorylation of p37 and GM130. As to p97/p47-mediated Golgi membrane fusion, we previously clarified that p47 phosphorylation on Serine-140 by Cdc2 results in mitotic inhibition of the p97/p47 pathway [11]. Together with our finding that VCIP135 is phosphorylated at mitosis, the mitotic inhibition of the p97/p37-mediated and p97/p47-mediated Golgi assembly are thought to be achieved in multiple ways; by the phosphorylation of GM130, p37 and VCIP135 in the p97/p37 pathway; and by the phosphorylation of p47 and VCIP135 in the p97/p47 pathway. These ternary and binary systems in the p97/p37 and the p97/p47 pathways, respectively, might enable very tight and precise control of Golgi disassembly–reassembly at mitosis.

On the other hand, VCIP135 was originally identified as a protein interacting with the p97/p47 complex: it binds to the p97/p47 complex and dissociates it via p97 catalyzed ATP hydrolysis [13]. Although VCIP135 also binds to p97 [13], it remains unclear whether complex formation between VCIP135 and p97 is really necessary for p97-mediated membrane fusion. In this study, we isolated a VCIP135 mutant, VCIP135(T760E, S767E), that still binds to WAC and maintains its deubiquitinating enzyme activity, but lacks p97-binding affinity. Our *in vitro* Golgi reassembly assay revealed that this mutant lacking p97-binding affinity does not function in either p97/p47-mediated nor p97/p37-mediated Golgi reassembly. Therefore, formation of the p97/VCIP135 complex is thought to be required for p97-mediated Golgi membrane fusion.

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