Identification of Phosphorylation Sites in the C-Terminal Region of Charged Multivesicular Body Protein 1A (CHMP1A)

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Note

Human charged multivesicular body protein 1A (CHMP1A) displayed two bands on SDS–PAGE and differences in efficiency of complex formation with IST1. By site-directed mutagenesis and phosphate-affinity PAGE, we identified Ser179 and Ser182 located in the C-terminal region as major phosphorylation sites that cause a mobility shift, but interaction with IST1 was not affected by Ser-to-Ala mutations.

Key words: ESCRT; CHMP; IST1; phosphorylation

Charged multivesicular body protein 1A (CHMP1A) is a member of the endosomal sorting complex required for transport-III (ESCRT-III). It has an Snf7 domain and a microtubule interacting and transport (MIT)-interacting motif (MIM) 1 in the C-terminal region (Fig. 1A, B). MIMs are recognized by proteins that contain the MIT domain such as VPS4s. The C-terminal region, including the MIM, regulates the open-closed conformation of CHMP proteins. In mammalian cells, CHMP1A localizes partially to the nucleus and to the cytoplasm in a punctate pattern, where it associates well with several proteins (nucleus, BMI1; cytoplasm, IST1 and calpain-7). We found that the autolytic activity of calpain-7 is enhanced by CHMP1B and by CHMP1A (unpublished data). In the present study, we analyzed the interaction between IST1 and CHMP1A by co-immunoprecipitation assay using HEK293T cells, in which IST1, fused with monomeric GFP at the N-terminus of IST1 (mGFP-IST1), was transiently co-expressed with wild-type (WT) CHMP1A tagged with FLAG at the N-terminus (FLAG-CHMP1A) or co-expressed with C-terminally truncated FLAG-CHMP1A1-172. We found that the CHMP1A proteins in the cleared cell lysates (input) were resolved into two bands by conventional SDS–PAGE followed by Western blot analysis (WB), in accord with previous studies. On the other hand, FLAG-CHMP1A1-172 showed only a single band (Fig. 1C, lower left panel). FLAG-CHMP1A, corresponding to the upper band, was exclusively co-immunoprecipitated with mGFP-IST1, and FLAG-CHMP1A1-172 was also co-immunoprecipitated (lower right panel). The appearance of doublet bands in WT but not in the C-terminally truncated mutant indicates that post-translational modifications such as limited proteolysis and phosphorylation occur in the C-terminal region. Since calpain-7 is an ESCRT-linked protease, there was a possibility that CHMP1A was cleaved by calpain-7, but neither overexpression nor knockdown of calpain-7 affected the electrophoretic mobility of FLAG-CHMP1A (data not shown).

Since there are multiple Ser residues in this region (Fig. 1B), we investigated the possibility of phosphorylation as cause of the appearance of doublet bands on SDS–PAGE. Treatment of the cleared cell lysates with calf intestine-alkaline phosphatase (CIAP) caused a convergence of the doublet FLAG-CHMP1AWT bands into one band corresponding to a lower band with the use of a conventional 12.5% gel (Fig. 2A, lanes 1 and 2). Since FLAG-CHMP1A1-172 showed a single band (Fig. 1C, lower left panel), phosphorylation sites should reside at amino acids from 173 to 196 of CHMP1A. To identify the phosphorylation sites or residues important in phosphorylation, we constructed expression vectors of Ser-to-Ala substitution mutants whose Ser residues were replaced with Ala at the corresponding sites in this region (S173A, S178A, S179A, S182A, and S188A) as well as at 166 (S166A) and all five Ser residues from 173 to 188 (5SA). The FLAG-CHMP1A1-172 mutant showed a single major band and a very faint more slowly migrating band in contrast to apparent doublet bands in the other single Ser-to-Ala mutants by conventional SDS–PAGE analysis of CIAP-untreated protein samples (Fig. 2A, odd lanes), suggesting that Ser179 is a major phosphorylation site. The signal intensity ratio of the upper band to the lower band of FLAG-CHMP1A1-172 was decreased compared to WT and the other mutants, suggesting that Ser182 was also phosphorylated.

To analyze phosphorylation of CHMP1A in more detail, FLAG-CHMP1A mutants were subjected to phosphate-affinity PAGE, in which phosphorylated proteins have been found to display much slower mobility. FLAG-CHMP1AWT was resolved into four bands before CIAP treatment and into one band after treatment (Fig. 2B, bands a–d), suggesting that phosphorylation occurred at several sites. The C-terminal deletion mutant (FLAG-CHMP1A1-172) showed a single band, as expected, confirming that phosphorylation occurs only in the C-terminal region. FLAG-CHMP1A1-172, -CHMP1A1-172, and -CHMP1A1-172 showed four bands in a pattern similar to WT, indicating

Abbreviations: CHMP1A, charged multivesicular body protein 1A; CIAP, calf intestine alkaline phosphatase; mAb, monoclonal antibody; MIT, microtubule interacting and transport; MIM, MIT-interacting motif; WB, Western blotting; WT, wild type

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that Ser\textsuperscript{166}, Ser\textsuperscript{178}, and Ser\textsuperscript{188} were not phosphorylated (Fig. 2C). On the other hand, FLAG-CHMP1A\textsuperscript{173/177A}, -CHMP1A\textsuperscript{179A}, and -CHMP1A\textsuperscript{S182A} showed fewer bands than WT. The bands that disappeared were: b in S173A; b, c, and d in S179A; and b and c in S182A, suggesting that Ser\textsuperscript{182} and Ser\textsuperscript{173} were also partially phosphorylated. The disappearance of bands b, c, and d due to single replacement of Ser\textsuperscript{179} with Ala suggests that Ser\textsuperscript{175} influences the efficiency of phosphorylation at neighboring Ser residues by protein kinases. A faint band (band e, indicated by an arrow in Fig. 2C), migrating faster than band a, appeared in the mutants except for mutations at Ser\textsuperscript{166} and Ser\textsuperscript{173}. Since band e was also observed even in the 5SA mutant (Ser\textsuperscript{173/177/179/182/188}, replaced with Ala), processing by limited proteolysis or other modifications in the C-terminal region might have been induced by the amino acid substitutions.

Since the phosphorylation of CHMP1A was assumed to control the affinity for IST1, we used phosphorylation-defective mutants of FLAG-CHMP1A in co-immunoprecipitation assays with mGFP-IST1. As shown in Fig. 2D, phosphate-affinity PAGE analysis of the immunoprecipitates revealed that some phosphorylated

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**Fig. 1.** Post-Translational Modification of CHMP1A in the C-Terminal Region. A, Schematic representation of CHMP1A. The core Snf7 domain is followed by a C-terminal region containing MIT-interacting motif 1 (MIM1). B, Amino acid sequence of the C-terminal region of CHMP1A. The sequence deleted in the truncation mutant used in this study and MIM1 are indicated by underline and asterisks respectively. Ser residues are shown in bold face. C, HEK293T cells were co-transfected with expression vectors for mGFP-fused IST1 and FLAG-tagged wild-type (WT) CHMP1A or FLAG-CHMP1A\textsuperscript{1-172}. After 24 h, the cells were lysed with buffer A (10 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 5 mM EDTA) supplemented with protease inhibitors (0.2 mM phenylmethylsulfonyl fluoride, 0.4 mM pefabloc, 6 μg/mL leupeptin, 2 μM E-64, 2 μM pepstatin). The cleared cell lysates (10,000 g supernatant, input) were incubated sequentially with rabbit anti-GFP antiserum or control rabbit IgG (Ctrl) for 2 h and with protein G-Sepharose 4 beads at 4°C. The cleared cell lysates obtained using buffer A (Input, left halves of panels) were washed 3 times with buffer A, and bound proteins (immunoprecipitates, IP) were subjected to SDS–PAGE, followed by Western blotting (WB) with an anti-FLAG monoclonal antibody (mAb) (upper panel) and with anti-FLAG mAb (lower panel). Asterisk, IgG light chain.

**Fig. 2.** Determination of Phosphorylation Sites in CHMP1A. At 24 h after HEK293T cells were transfected with expression plasmids for wild-type (WT) FLAG-CHMP1A, single Ser-to-Ala substitution mutants (S166A, S173A, S178A, S179A, S182A, and S188A), a five-Ser-to-Ala substitution mutant (Ser\textsuperscript{S166A/S173A/S178A/S179A/S182A/188A}), and C-terminal deletion mutant (1–172), cells were harvested and lysed with buffer B (10 mM HEPES–NaOH, pH 7.4, 142.5 mM KCl, 0.2% NP-40) supplemented with protease inhibitors. After sonication, the lysates (20 μL) were mixed with an equal volume of buffer C (100 mM Tris–HCl, pH 8.5, 0.2 mM Zn(OAc)\textsubscript{2}, 2 mM MgCl\textsubscript{2}) and incubated with 20 units of calf intestine alkaline phosphatase (CIAP, Takara, Otsu, Japan) for 4 h. The samples untreated with CIAP contained 10 mM sodium fluoride, 5 mM sodium pyrophosphate, 10 mM β-glycerophosphate, and 2 mM sodium orthovanadate. Protein samples were resolved by conventional SDS–PAGE on a 12.5% gel (A) or on a 17.5% Anderson gel containing Phos-tag acrylamide (Wako Pure Chemicals, Osaka) (B),\textsuperscript{3} followed by WB with an anti-FLAG mAb. Multiple bands detected by WB in (B) are labeled a to d. C, Samples untreated with CIAP were analyzed as in (B). A faint band not found in WT is indicated by an arrow, and was named band e. HEK293T cells were transfected with pmGFP-IST1, pFLAG-CHMP1A, and the mutants as indicated above the panels. At 24 h after transfection, cleared cell lysates obtained using buffer A (Input, left halves of panels) were subjected to immunoprecipitation (IP, right halves of panels) with an anti-FLAG serum followed by conventional (upper panel) or Phos-tag SDS–PAGE (lower panel). WB was carried out with an anti-FLAG mAb and with an anti-FLAG mAb to detect mGFP-IST1 (upper panel) and WT and mutant proteins of FLAG-CHMP1A (lower panel) respectively.

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FLAG-CHMP1A proteins efficiently bound to mGFP-IST1 (Fig. 2D, band b, lanes 7, 8, and 12; band c, lane 9; and band d, lanes 9 and 11). However, presumably unphosphorylated proteins corresponding to band a of FLAG-CHMP1A<sup>S173A</sup>, FLAG-CHMP1A<sup>S178,179A</sup>, and FLAG-CHMP1A<sup>S182A</sup> showed binding to mGFP-IST1 (lanes 9, 10, and 11). Since the C-terminal region of CHMP proteins regulates an open-closed conformation and influences dimerization and association with other CHMP proteins, replacement of Ser with Ala in this region might change the local structure of CHMP1A that leads to derepression of the autoinhibitory C-terminal region and favors interaction with IST1.

In this study, we identified phosphorylation sites in CHMP1A for the first time. Although no functional relationships of phosphorylation were found in IST1-binding, the knowledge obtained about phosphorylation might add to recent genetic and physiological studies that indicate the involvement of CHMP1A in tumor suppression and cerebellar development.5,11)

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