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Deubiquitination of Chfr, a checkpoint protein, by USP7/HAUSP regulates its stability and activity

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Abstract

Chfr, a mitotic stress checkpoint, plays an important role in cell cycle progression, tumor suppression and the processes that require the E3 ubiquitin ligase activity mediated by the RING finger domain. Chfr stimulates the formation of polyubiquitin chains by ub-conjugating enzymes, and induces the proteasome-dependent degradation of a number of cellular proteins including Plk1 and Aurora A. In this study, we identified USP7 (also known as HAUSP), which is a member of a family of proteins that cleave polyubiquitin chains and/or ubiquitin precursors, as an interacting protein with Chfr by immunoaffinity purification and mass spectrometry, and their interaction greatly increases the stability of Chfr. In fact, USP7 can remove ubiquitin moiety from the autoubiquitinated Chfr both *in vivo* and *in vitro*, which results in the accumulation of Chfr in the cell. Thus, our finding suggests that USP7-mediated deubiquitination of Chfr leads to its accumulation, which might be a key regulatory step for Chfr activation and that USP7 may play an important role in the regulation of Chfr-mediated cellular processes including cell cycle progression and tumor suppression.

Keywords: Chfr; USP7; HAUSP; Ubiquitination; Deubiquitination; Mass spectrometry

Ubiquitin-dependent proteolysis plays a crucial role in the regulation of a diverse cellular processes, including cell proliferation, differentiation, and apoptosis [1]. Ubiquitin, a 76-amino-acid polypeptide, is covalently attached to target proteins by a cascade system consisting of Ub-activating (E1), conjugating (E2), and ligating (E3) enzymes [2]. E3 Ub-ligases that confer the substrate specificity have been grouped into two families; the HECT-domain family that is defined by its homology to the C-terminus of E6-associated protein (E6AP) and the RING family carrying RING-H2 domain that is essential for the Ub-ligase activity [3].

Chfr (checkpoint protein with FHA and RING domains), a RING family Ub-ligase, was identified as defining a mitotic checkpoint that delays transition to metaphase in response to mitotic stress [4]. Chfr contains an N-terminal FHA domain, which is involved in phosphoprotein interaction [5,6], and a RING finger domain, which participates in protein ubiquitination [7]. The RING finger domain in Chfr is required for the ligase activity and for autoubiquitination [8]. Chfr plays an important role in cell cycle progression and tumor suppression as a stress checkpoint, and ensures chromosomal stability by controlling the expression levels of key mitotic proteins such as Plk1 and Aurora A [9–11]. Although Chfr is ubiquitously expressed in normal tissues, it is frequently downregulated in human cancers [4], mostly owing to hypermethylation of its promoter region [12].

In general, polyubiquitinated proteins are targeted for degradation by the 26S proteasome. However, deubiquitination, a removal process of ubiquitin from ubiquitin-conjugated protein substrates, is mediated by deubiquitinating enzymes, which controls the cellular levels of substrate proteins [13,14]. Ubiquitination and deubiquitination have a precise role for selective protein degradation in eukaryotic

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cells, and are important for the regulation of a number of intracellular processes including cell cycle, apoptosis, transcriptional activation, signal transduction, antigen presentation, oncogenesis, preimplantation, and DNA repair [2,14,15]. For example, p53 stability is regulated by deubiquitination mediated by herpesvirus-associated ubiquitin-specific protease known as HAUSP (also known as human USP7), which directly deubiquitinates and stabilizes p53 [16]. USP7 also plays a crucial role in the regulation of p53-dependent apoptosis and the inhibition of cell growth [16]. It has been shown that a partial reduction of endogenous USP7 levels destabilizes endogenous p53 [17]. However, nearly complete ablation of USP7 stabilizes and activates p53 because USP7 is also required for the stability of endogenous MDM2, which is constitutively self-ubiquitinated and degraded *in vivo* [18]. In the absence of USP7, MDM2 appears to be extremely unstable, leading to indirect p53 activation [18]. This feedback-mediated p53 stabilization is MDM2-dependent [16,17].

In the present study, we demonstrate that Chfr binds to the ubiquitin-specific protease USP7 and their interaction greatly increase the stability of Chfr. Moreover, USP7 can remove ubiquitin moiety from the autoubiquitinated Chfr both *in vivo* and *in vitro*, which results in preventing the Chfr degradation. Taken together, we suggest that USP7-mediated deubiquitination of Chfr leads to its accumulation, which might be function as a signal for Chfr activation and that USP7 may play an important role in the regulation of Chfr-mediated cellular processes including cell cycle progression and tumor suppression.

Materials and methods

Plasmids and cells. A full-length *CHFR* cDNA was amplified by polymerase chain reaction (PCR) from Marathon-Ready human brain cDNA (Clontech), and subcloned into p3xFLAG-CMV10 (Sigma) or pcDNA4-HisMax (Invitrogen) vector. *USP7* was obtained from Dr. C.H. Chung (Seoul National University), and cloned into p3xFLAG-CMV10 and pcDNA4-HisMax. HEK293T and HeLa cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (HyClone), 100 μg/ml streptomycin and 100 U/ml penicillin (HyClone) at 37 °C in a humidified 5% CO_2 atmosphere. Transient transfections were performed using Lipofectamine Plus (Invitrogen) according to the manufacturer's instruction.

Immunoprecipitation and immunoblot. For immunoprecipitation, cells were lysed in 20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 0.1 mM EDTA, 0.2% Triton X-100, and 1× protease inhibitor mixture (Roche Applied Science). Cell lysates were incubated with anti-M2 resin (Sigma) for 1.5 h at 4 °C. The resins were collected by centrifugation and washed three times with buffer consisting of 20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 0.1 mM EDTA, and 1.0% Triton X-100. Bound proteins were eluted by 0.1% SDS in 50 mM Tris–HCl (pH 8.0) and subjected to SDS–PAGE followed by stain with silver or immunoblot with anti-HA antibody (Santa Cruz Biotechnology), anti-Xpress antibody (Invitrogen), anti-Ub antibody (BostonBiochem), anti-FLAG antibody, anti-His antibody and anti-β-actin antibody (Sigma).

Assays for ubiquitination/deubiquitination. For *in vivo* ubiquitination assays, HEK293T cells were transfected with plasmids encoding FLAG-Chfr, HA-Ub, and HisMax-USP7. At 24 h after transfection, cells were treated with 2 μM MG132 (Sigma) for 12 h. Cells were lysed in 20 mM Tris–HCl (pH 7.5) buffer containing 150 mM NaCl, 0.1 mM EDTA, 0.2% Triton X-100 and 1× protease inhibitor mixture. Cell lysates were then incubated with anti-M2 resin for 2 h at 4 °C. After wash the resin with the same buffer, the proteins bound to resin were eluted by SDS-sampling buffer and subjected to SDS–PAGE followed by immunoblot with anti-HA antibody. For *in vitro* ubiquitination assays, the purified His-Chfr (1 μg) from insect cells was incubated at 37 °C for 30 min with E1 (0.5 μg), Ubc5 (0.5 μg), Ub (5 μg) and an ATP-regenerating system (50 mM Tris–HCl at pH 7.5, 5 mM $MgCl_2$, 10 mM creatine phosphate, 5 U/ml phosphocreatine kinase, and 5 mM ATP). After incubation, the samples were incubated 37 °C for further 60 min in the absence or presence of FLAG-USP7 purified from HEK293T cells.

Results and discussion

USP7 is the major interacting protein with Chfr

To identify proteins that interact with the Chfr proteins, we carried out immunoaffinity purification in combination with mass spectrometry. HeLa cells were transiently transfected with pCMV-3xFLAG as a control or pCMV-3xFLAG-Chfr/I306A, which lacks ubiquitin ligase activity by replacement of Ile306 with Ala. The Chfr immunoprecipitate, but not the immunoprecipitate from control transfected cells, contained several proteins including the 135 kDa polypeptides (Fig. 1A). After a large preparation, enough material of the 135 kDa band was obtained for mass spectrometry; three peptide sequences were obtained, all of which were derived from the herpesvirus-associated ubiquitin-specific protease known as HAUSP (also known as human ubiquitin-specific protease 7, USP7) [19]. To further evaluate the interaction between Chfr and USP7, HEK293T cells were then transfected with HisMax-tagged Chfr and/or FLAG-tagged USP7. As shown in Fig. 1B, Chfr was readily immunoprecipitated from the cell transfected with both Chfr and USP7 but not from the cells transfected with Chfr alone. Furthermore, it was previously reported that either Chfr or USP7 are localized in PML body [19,20]. Taken together, these results indicate that USP7 interacts with Chfr *in vivo* and that the possible regulation of Chfr by USP7 might be effective in the cells.

USP7 regulates the Chfr stability

Chfr is a RING finger ubiquitin ligase which can be regulated by autoubiquitination and subsequent degradation [8,15], and USP7 has been shown to bind to the many of RING finger ubiquitin ligases and thereby protects them from autoubiquitination and subsequent degradation [15]. To address the question that USP7 can protect the degradation of Chfr, we examined the protein levels of Chfr in HEK293T cells expressing USP7. Western blot analysis showed that the level of Chfr in cells expressing HA-Ub is much less than that seen in control cells, whereas the level of Chfr in cells expressing both of USP7 and HA-Ub was markedly increased (Fig. 2A). Notably, the level of Chfr in cells expressing HA-Ub only markedly diminished compare to that in cells missing HA-Ub, which is likely to be the increase of autoubiquitination by Chfr

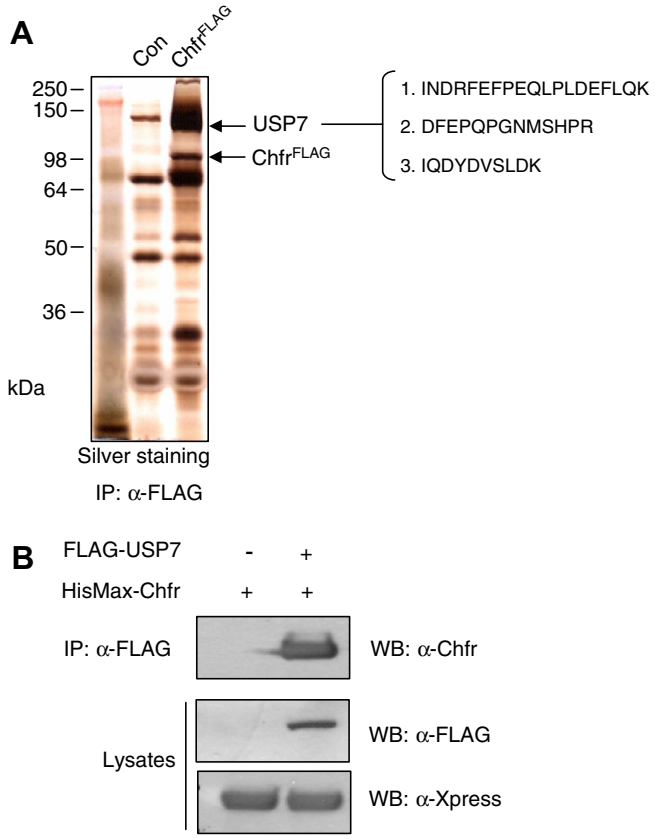

Fig. 1. Purification of USP7, and interactions between Chfr and USP7. (A) Identification of USP7 as a novel Chfr-binding protein. HeLa cells were transiently transfected with pCMV-3XFLAG or pCMV-3XFLAG-Chfr/I306A, and cell lysates were immunoprecipitated with anti-M2 resin. Proteins coimmunoprecipitating with FLAG-Chfr were separated by SDS–PAGE, stained with silver. Peptide sequences derived from the 135 kDa protein band were obtained by mass spectrometry. (B) Interaction between Chfr and USP7 proteins. FLAG-tagged USP7 was transfected to HEK293T cells with HisMax-tagged Chfr. Cell lysates were immunoprecipitated (IP) with anti-M2 resin and immunoblotted (WB) with indicated antibodies.

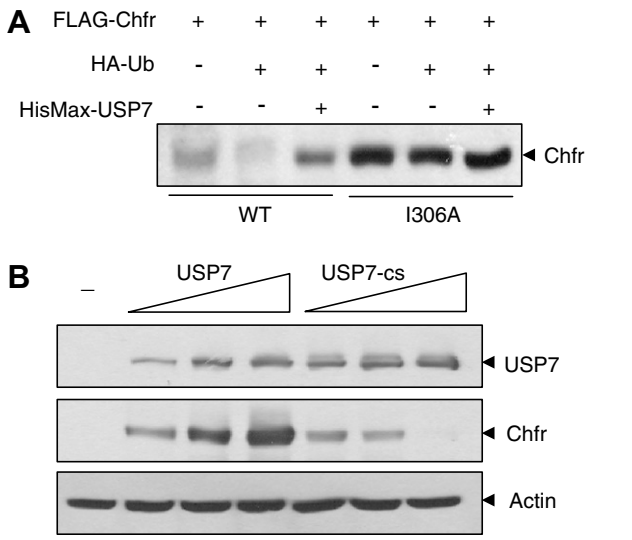

Fig. 2. USP7 increases the stability of Chfr *in vivo*. (A) Expression vector encoding HisMax-tagged USP7 was transfected to HEK293T cells with different combination of HA-Ub, FLAG-Chfr (wild-type), and FLAG-Chfr/I306A mutant. Cell lysates were prepared and subjected to immunoblot analysis with anti-FLAG antibody. (B) Plasmid encoding FLAG-Chfr and HA-Ub were transfected to HEK293T cells with increasing amounts of vectors encoding HisMax-USP7 (wild-type) or HisMax-USP7-cs mutant. Cell lysates were then prepared and subjected to immunoblot analysis with anti-FLAG (for Chfr), anti-Xpress (for USP7), and anti-β-actin antibodies.

itself. On the other hand, Chfr/I306A that lacks ubiquitin ligase activity is more stable than the wild-type (WT) protein, and its level was not affected by expressing both of HA-Ub and USP7, indicating that the ubiquitin ligase activity of Chfr contributes to its own turnover. These results implicate that the level of Chfr is autoregulated by its ubiquitin ligase activity and that USP7 may play an important role in regulating the stability of Chfr *in vivo*. Next, to clarify the protective effect of USP7 on the Chfr stability *in vivo*, HEK293T cells expressing Chfr were transfected with plasmid encoding wild-type USP7 or mutant USP7-cs. USP7-cs is a dominant negative mutant which a highly conserved Cys residue at the core domain was replaced by Ser [16]. As shown in Fig. 2B, the protein level of Chfr was gradually accumulated by the increment of USP7, while in case that USP7-cs instead of wild-type USP7 was increased, the level of Chfr was rather decreased. This result implicates that deubiquitination activity of USP7 is crucial for the regulation of the protein level of Chfr, especially considering that USP7-cs mutant was still able to interact with Chfr (data not shown). And it looks like that Chfr did not affect the cellular level of USP7, suggesting that Chfr may not contribute to USP7 protein turnover. Taken together, these results clearly indicate that USP7 binds to Chfr and increases the stability of Chfr *in vivo*.

USP7 deubiquitinates Chfr in vivo and in vitro

USP7 is known as a ubiquitin-specific isopeptidase which can remove ubiquitin moiety from the polyubiquitinated proteins such as p53 and thereby plays a crucial role on p53 stabilization [15,17]. Thus, we examined whether USP7 can mediate the deubiquitination of Chfr *in vivo*. FLAG-tagged Chfr was transfected with HisMax-tagged USP7 and HA-Ub. Co-expression of Chfr with HA-Ub significantly enhanced the autoubiquitination of Chfr, whereas the ubiquitination of Chfr in cells expressing USP7 markedly diminished (Fig. 3A). Next, to examine whether USP7 affects the accumulation of ubiquitinated Chfr *in vitro*, we carried out the ubiquitination/deubiquitination assay using His-Chfr isolated from insect cells and FLAG-USP7 purified from HEK293T cells. Western blot analysis showed that autoubiquitination of Chfr was significantly promoted by incubation with E2 ub-conjugating enzyme, UbcH5, and Ubiquitin. On the other hand, little or no accumulation of ubiquitinated Chfr was observed in the presence of USP7 (Fig. 3B). Moreover, accumulation

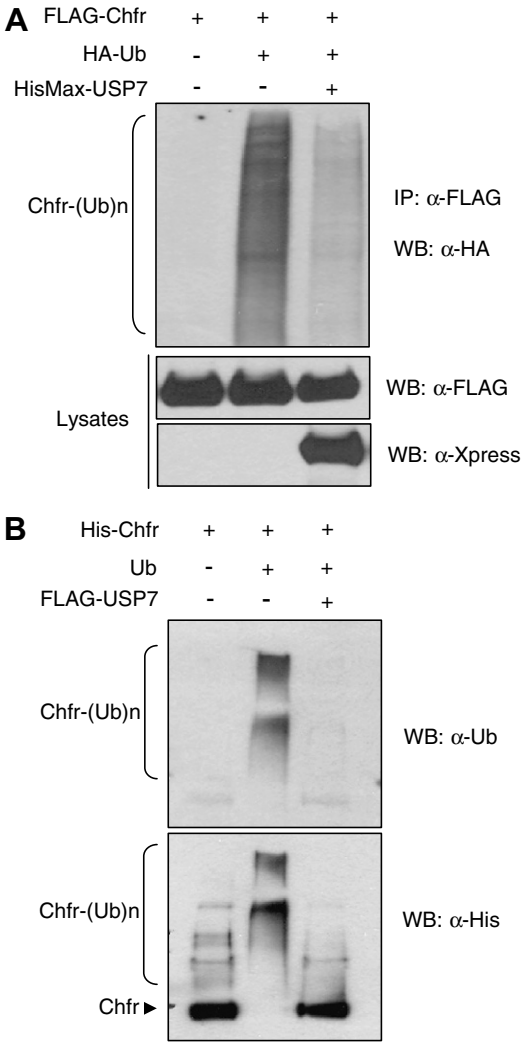

Fig. 3. USP7 mediates deubiquitination of Chfr. (A) Chfr was deubiquitinated in cells expressing USP7. FLAG-Chfr was transfected to HEK293T cells with different combination of HisMax-USP7 and HA-Ub. At 24 h after transfection, cells were treated with MG132 (2 μM) for 12 h, and cell lysates were prepared, immunoprecipitated with anti-M2 resin, and subjected to immunoblot analysis with anti-HA antibody. To evaluate the expression of USP7 or Chfr, cell lysates were immunoblot with anti-FLAG (for Chfr) and anti-Xpress (for USP7) antibodies. (B) USP7-mediated deubiquitination of Chfr *in vitro*. His-Chfr purified from insect cells were incubated at 37 °C for 30 min with E1, E2 (UbcH5), and Ubiquitin. After incubation, the samples were incubated 37 °C for further 60 min in the absence or presence of FLAG-USP7 purified from HEK293T cells. The reactions were then subjected to SDS–PAGE and immunoblot with anti-Ub or anti-His (for Chfr) antibody.

of ubiquitinated Chfr was not affected even in the presence of USP7-cs (data not shown), which is consistent with the finding that USP7-cs did not affect the stability of Chfr *in vivo* (see Fig. 2B). These results demonstrate that USP7 preferentially functions in deubiquitination of Chfr, and prevents autoubiquitination-mediated degradation of Chfr.

The present study has demonstrated that Chfr binds to the ubiquitin-specific protease USP7, a member of a family of proteins that cleave polyubiquitin chains and/or ubiquitin precursors, and their interaction greatly increases the stability of Chfr. Furthermore, USP7 can remove ubiquitin moiety from the autoubiquitinated Chfr both *in vivo* and *in vitro*, which results in preventing the Chfr degradation. Thus, our finding implicates that USP7-mediated deubiquitination of Chfr leads to its accumulation in the cell, which might be one of the regulatory mechanism toward Chfr activation, and that USP7 may play an important role in the regulation of Chfr-mediated cellular processes including cell cycle progression and tumor suppression.

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