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Molecules and Cells

Nuclear Localization of Chfr Is Crucial for Its Checkpoint Function

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Chfr, a checkpoint with FHA and RING finger domains, plays an important role in cell cycle progression and tumor suppression. Chfr possesses the E3 ubiquitin ligase activity and 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. While Chfr is a nuclear protein that functions within the cell nucleus, how Chfr is localized in the nucleus has not been clearly demonstrated. Here, we show that nuclear localization of Chfr is mediated by nuclear localization signal (NLS) sequences. To reveal the signal sequences responsible for nuclear localization, a short lysine-rich stretch (KKK) at amino acid residues 257-259 was replaced with alanine, which completely abolished nuclear localization. Moreover, we show that nuclear localization of Chfr is essential for its checkpoint function but not for its stability. Thus, our results suggest that NLS-mediated nuclear localization of Chfr leads to its accumulation within the nucleus, which may be important in the regulation of Chfr activation and Chfr-mediated cellular processes, including cell cycle progression and tumor suppression.

INTRODUCTION

The ubiquitin-proteasome system controls the levels of many cellular proteins via a cascade of enzymes termed E1, E2, and E3, which activate and conjugate ubiquitin to its substrates and proteasome-dependent degradation (Hershko and Ciechanover, 1998). The E3 enzymes, Ub-ligases, catalyze ubiquitin conjugation in a substrate-specific manner. The E3 enzymes can be grouped into two classes, depending on the presence of either a HECT domain or a RING domain. The RING domain provides a docking site for E2 enzymes that exhibit catalytic activity, whereas the HECT domain forms a catalytically essential thioester with ubiquitin (Pickart, 2001).

The Chfr (checkpoint with FHA and RING finger domains) protein, a RING-type E3 ligase, is a mitotic checkpoint regulator that arrests cells in early prophase in the presence of microtubule poisons (Scolnick and Halazonetis, 2000). Chfr contains an N-terminal FHA domain involved in phosphoprotein interaction (Stavridi et al., 2002) and a RING finger domain required for ubiquitin ligase activity that functions by recruiting ubiquitin-conjugating enzymes and for its autoubiquitination (Joazeiro et al., 1999; Oh et al., 2007). Chfr plays an important role in cell cycle progression and tumor suppression by functioning as a stress checkpoint regulator and ensures chromosomal stability by controlling cellular levels of key mitotic proteins such as Plk1 and Aurora A (Kang et al., 2002). Although Chfr is ubiquitously expressed in normal tissues, it is frequently down-regulated in human cancers, largely due to hypermethylation of its promoter region (Corn et al., 2003; Mizuno et al., 2002; Scolnick and Halazonetis, 2000; Shibata et al., 2002). A recent study showed that Chfr binds to the ubiquitin-specific protease USP7/HAUSP, and their interaction greatly increases the stability of Chfr. USP7 can remove the ubiquitin moiety from the autoubiquitinated Chfr, thereby preventing degradation of Chfr. USP7-mediated deubiquitination of Chfr leads to its accumulation, which may function as a signal for Chfr activation. Through this mechanism, USP7 may play an important role in the regulation of Chfr-mediated cellular processes (Oh et al., 2007).

In eukaryotic cells, the genome and transcriptional machinery in the nucleus are separated from the translational machinery in the cytoplasm; therefore, appropriate transport systems are required between these discrete compartments. All macromolecules enter and exit the nucleus via nuclear pore complexes embedded in the nuclear envelope (Suntharalingam and Wente, 2003; Tran and Wente, 2006) and require specific signal sequences to transport between the nucleus and cytoplasm (Lange et al., 2007). Nuclear targeting signals consist of stretches of basic amino acids (Dingwall and Laskey, 1991; Robbins et al., 1991) that are recognized by nuclear transport receptors termed importins (Gorlich and Kutay, 1999). Previous study showed that Chfr, which is localized mainly in the cell nucleus, can be recruited to the PML-nuclear body (PML-NB) through SUMO-1 modified PML (Daniels et al., 2004). However, the exact nuclear targeting sequences that enable this protein transport are currently unknown.

In the present study, we conducted domain localization analysis and mutagenesis to elucidate the potential signal sequence responsible for nuclear localization of Chfr. Our results clearly defined NLS sequence is responsible for Chfr nuclear localization and nuclear localization of Chfr is important for its cellular function.

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MATERIALS AND METHODS

Plasmids and cells

CHFR cDNA obtained from human brain cDNA library was amplified by PCR and cloned into the p3X-FLAG-CMV10 (Sigma) vector. Site-directed mutagenesis was conducted using the QuikChange Kit (Stratagene). HeLa cells were grown in DMEM supplemented with 100 units/ml penicillin, 1 μg/ml streptomycin, and 10% FBS at 37°C. Transient transfections were carried out using Lipofectamine Plus (Invitrogen) according to the manufacturer's instructions.

Immunoprecipitation and immunoblotting

For immunoprecipitation, cells were lysed in 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1 mM EDTA, and 0.2% Triton X-100 and 1× protease inhibitor cocktail (Roche Applied Science). Cell lysates were incubated with anti-M2 resin (Sigma) for 2 h at 4°C. Resins were collected by centrifugation and washed 3 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 0.2% SDS and separated by SDS-PAGE, followed by immunoblotting with appropriate antibodies (Byun and Jung, 2008). The following antibodies were used: anti-FLAG (Sigma), anti-myc and anti-HA (Santa Cruz Biotechnology), anti-GAPDH (Cell Signaling Technology), peroxidase-conjugated AffiniPure goat anti-rabbit and anti-mouse IgGs (Bio-Rad), and rabbit polyclonal anti-Chfr antiserum (raised against a recombinant His-Chfr).

Assays for ubiquitination

For *in vivo* ubiquitination assays, HeLa cells were transfected with appropriate expression vectors. Twenty-four hours after transfection, the cells were treated with 2 μM MG132 (Sigma) for 12 h. Cells were lysed in 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1 mM EDTA, and 0.2% Triton X-100 and 1× protease inhibitor cocktail and incubated with indicated antibodies for 2 h at 4°C. The resins were collected by centrifugation and washed 3 times in 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 using 0.2% SDS and separated by SDS-PAGE, followed by immunoblotting with anti-HA antibody.

Immunocytochemistry

HeLa cells were grown on glass cover slips. The cells were fixed with 2% formaldehyde in PBS for 30 min at room temperature and permeabilized with 0.5% Triton X-100 in PBS. All subsequent dilutions and washes were performed with PBS containing 0.1% Triton X-100 (PBS-T). Nonspecific binding sites were saturated by incubation for 30 min with a blocking solution consisting of 10% goat serum and 1% gelatin in PBS. Cells were incubated with primary antibody for 1 h and washed 4 times with PBS-T at 10-min intervals. The cells were then incubated with FITC- or TRITC-conjugated secondary antibody for 1 h and washed 4 times. DAPI was used for counterstaining cell nuclei. Vectashield was used to mount glass cover slips, and the cells were visualized under a Zeiss Axioplan II microscope.

Luciferase reporter assay

HeLa cells were transfected with appropriate expression vectors. After incubation for 48 h, the cells were lysed and processed for luciferase and β-galactosidase assays in a luminometer. Luciferase activity was determined and normalized to the co-transfected β-galactosidase activity with a luciferase system (Promega). Values are expressed as mean ± s.d. for at least 3 independent experiments.

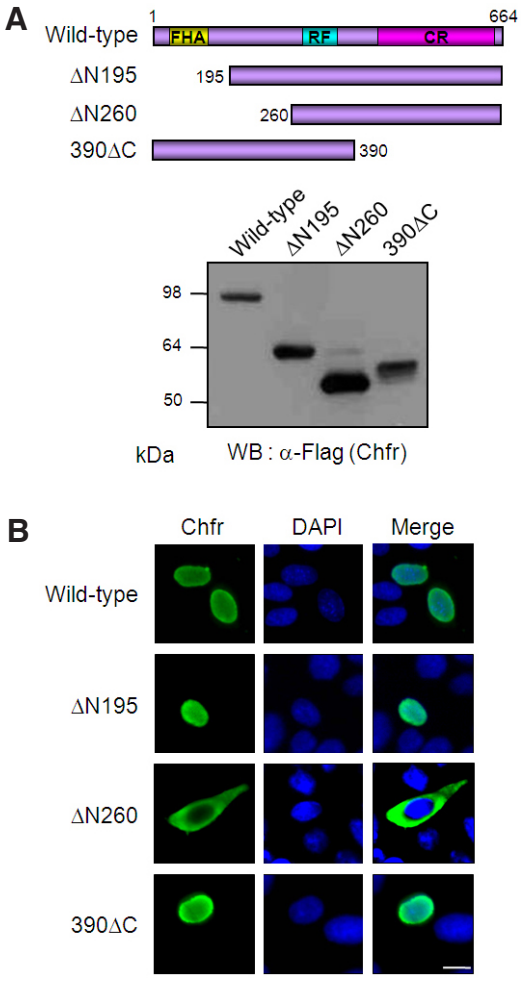

Fig. 1. Identification of Chfr nuclear localization domains. (A) Schematic representation of Chfr domain structure. WT, wild-type; ΔN195, aa 195-664 fragment; ΔN260, aa 260-664 fragment; 390ΔC, aa 1-390 fragment. FHA, forkhead-associated domain; RF, RING finger domain; CR, cysteine-rich domain. All constructs were Flag-tagged. HeLa cells were transfected with Flag-Chfr wild-type and deletion mutants. After incubation for 48 h, cell lysates were separated by SDS-PAGE, followed by immunoblotting with anti-Flag antibody. (B) Localization of wild-type or mutant forms of the Chfr protein was detected by immunofluorescence analyses. HeLa cells were stained with anti-Flag antibody (green) and DAPI (blue). Scale bars: 10 μm.

RESULTS

Identification of Chfr nuclear localization signal sequence

To identify domains responsible for nuclear localization of Chfr, the subcellular distribution of several Chfr fragments was determined. Flag-tagged deletion mutants of Chfr were generated and transiently expressed in HeLa cells. Western blot analysis and immunocytochemistry were performed with these mutants (Fig. 1). As expected, full-length Chfr was localized predominantly in the nucleus. While peptide fragments containing amino acid (aa) residues 195-664 and 1-390 were localized in the nucleus, a fragment containing aa residues 260-664 was

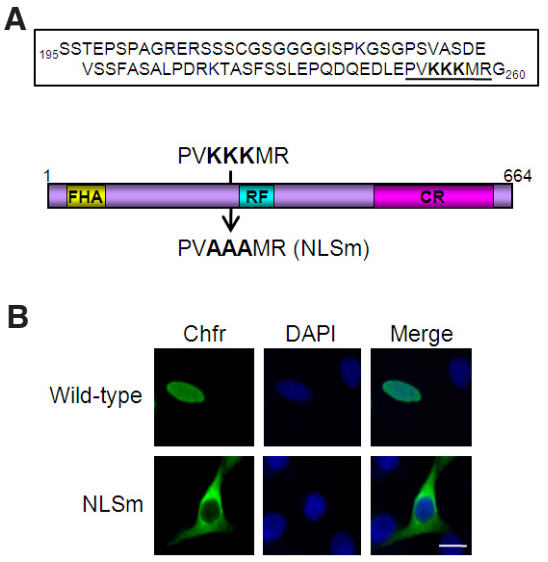

Fig. 2. Site-directed mutagenesis of full-length Chfr reveals the involvement of NLS sequences in nuclear localization. (A) The Chfr sequence corresponding to amino acids 195-260 and site-directed mutations of potential NLS. The lysine residues that were changed to alanine to create Chfr NLSm are indicated in bold. (B) Immunofluorescence analyses of the K257A/K258A/K259A mutant (NLSm). Cells were stained with anti-Flag antibody (green) and DAPI (blue). Scale bars: 10 μm.

localized in the cytoplasm. These results suggest that the Chfr region consisting of aa residues 195-260 is required for its nuclear localization. We then investigated putative NLS sequences within the Chfr region of aa residues 195-260. A classical NLS consists of a short sequence enriched in basic amino acids (Dingwall and Laskey, 1991; Robbins et al., 1991). Chfr contains a short lysine-rich stretch in the peptide region corresponding to aa residues 195-260. As shown in Fig. 2, the predicted NLS at residues 257-259, KKK, was replaced with AAA (referred to as NLSm), which completely abolished nuclear localization of Chfr. These results suggest that the KKK sequence located at aa positions 257-259 is indeed a functional nuclear localization signal sequence.

Nuclear localization of Chfr is not essential for its Ub-ligase activity and stability

Chfr is a RING-finger ubiquitin ligase that can be regulated by autoubiquitination, which promotes subsequent degradation. To determine whether nuclear localization is important for its Ub-ligase activity, we examined the level of autoubiquitination of wild-type and NLSm Chfr in HeLa cells expressing Chfr and/or ubiquitin. Western blot analysis showed that both wild-type and NLSm Chfr are autoubiquitinated efficiently (Fig. 3A). These data suggest that nuclear localization of Chfr is not essential for its autoubiquitination. We then assessed the stability of wild-type and NLSm Chfr by monitoring protein levels by western blot analysis after treatment of cells with cycloheximide to inhibit protein synthesis. Time-course measurements for Chfr degradation revealed no obvious difference in stability between wild-type and NLSm Chfr proteins (Fig. 3B).

Nuclear localization of Chfr is required for p21 expression

Since the Chfr NLSm mutant did not localize to the nucleus, we hypothesized that it may not be functional for its nuclear target.

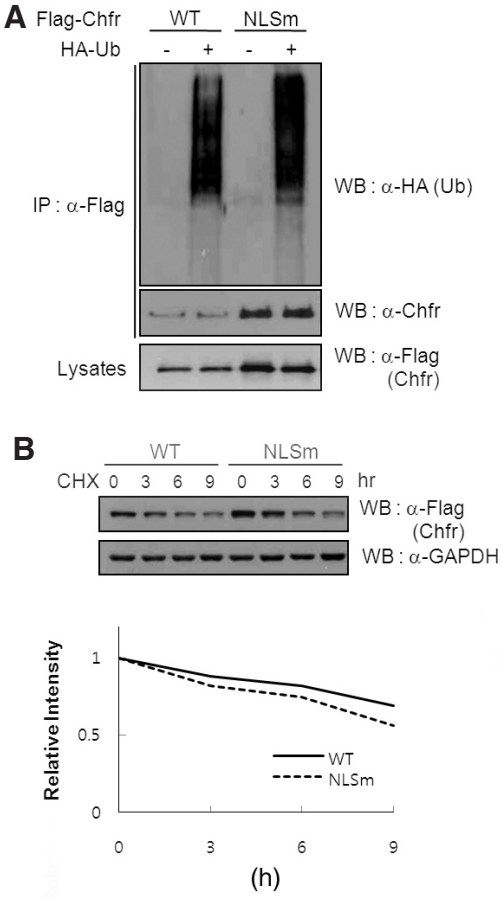

Fig. 3. Nuclear localization of Chfr is not essential for its Ub-ligase activity and stability. (A) HeLa cells were co-transfected with Flag-Chfr (wild-type, NLSm) and/or HA-ub. Cell lysates were immunoprecipitated with anti-M2 resin, and precipitates were subjected to SDS-PAGE, followed by immunoblotting with anti-Flag or anti-Chfr antibody. (B) HeLa cells were transfected with Flag-Chfr (wild-type, NLSm). At 24 h after transfection, the cells were treated with 200 μg/ml cycloheximide for 9 h. Cell lysates were then subjected to SDS-PAGE, followed by immunoblotting with anti-Flag or anti-GAPDH antibody. Relative band intensity was quantified by densitometry using ImageJ software.

Recently, Chfr was shown to be involved in downregulation of HDAC1 by promoting its ubiquitination and proteasome-dependent degradation, which results in the accumulation of the CDK inhibitor $p21^{CIP1/WAF1}$ (Oh et al., 2009). We tested whether the NLS mutation affects the levels of HDAC1. As shown in Fig. 4A, wild-type Chfr expression, but not NLSm promotes downregulation of HDAC1. To determine whether NLSm mutant has Ub-ligase activity on HDAC1, myc-tagged HDAC1 was co-expressed either with Chfr wild-type, NLSm, or I306A [lacking Ub-ligase activity (Kang et al., 2002)] in HeLa cells. Cell lysates were then prepared and subjected to immunoprecipitation with anti-myc resin, followed by immunoblotting with anti-HA antibody. As shown in Fig. 4B, wild-type Chfr was associated with significant ubiquitination of HDAC1. In contrast, little or no ubiquitination of HDAC1 was observed in cells expressing NLSm or I306A Chfr proteins. Since Chfr-mediated degradation of HDAC1 promotes acetylation of p21 (Ocker and Schneider-

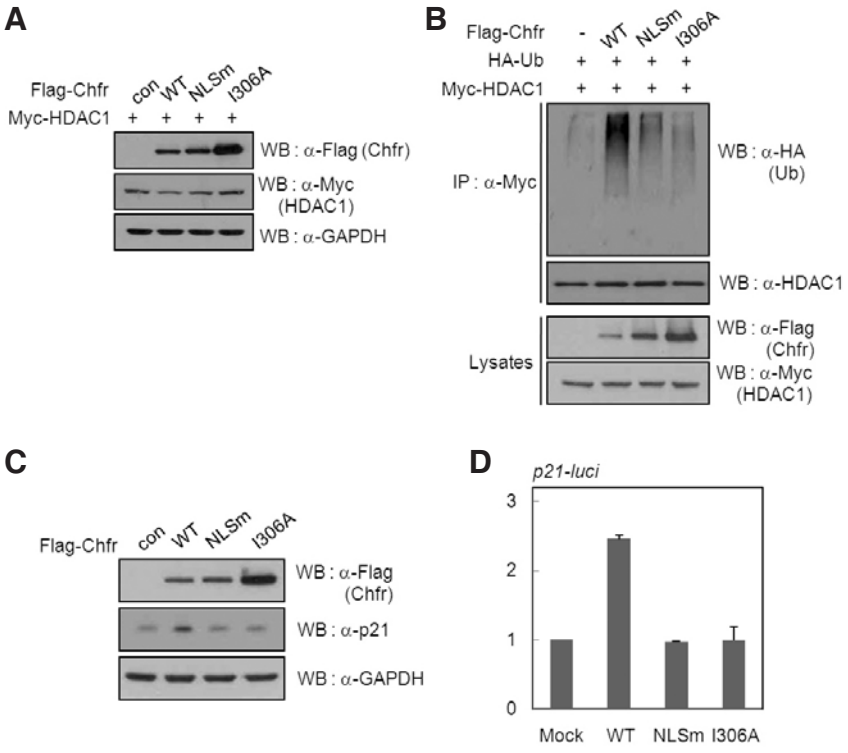

Fig. 4. Nuclear localization of Chfr is involved in the regulation of HDAC1 and p21. (A) Myc-HDAC1 (0.5 μg) was co-transfected into HeLa cells with 2 μg of Chfr wild-type or mutants. Cell lysates were subjected to SDS-PAGE, followed by immunoblotting with anti-Flag, anti-myc, or anti-GAPDH antibody. (B) HeLa cells were transfected with Flag-Chfr (empty vector, wild-type, NLSm, or I306A), HA-Ub, and Myc-HDAC1. After incubation for 24 h, the cells were treated with 2 μM MG132 for 12 h. Cell lysates were immunoprecipitated with anti-myc resin and precipitates were subjected to SDS-PAGE followed by immunoblotting with anti-HA and anti-Chfr antibody. (C) Wild-type Chfr or mutants (2 μg) were transfected into HeLa cells. After 48 h incubation, cell lysates were analyzed by western blot analysis with anti-Flag, anti-p21, or anti-GAPDH antibody. (D) HeLa cells were transfected with Chfr wild-type or mutants. After 48 h incubation, luciferase activity was measured by a luminometer and normalized by β-galactosidase assay. Values are expressed as mean ± s.d. for 3 independent experiments (n = 3).

Stock, 2007; Oh et al., 2009), we examined whether NLSm Chfr affects p21 protein levels. As shown in Fig. 4C, endogenous p21 protein levels were increased by wild-type Chfr but not by NLSm or I306A mutant forms. To evaluate the effects of wild-type and mutant Chfr on transcriptional regulation by HDAC1, a *p21*-luciferase reporter gene was employed. Wild-type Chfr enhanced expression of the reporter gene, while NLSm and I306A Chfr had no effect (Fig. 4D). These results indicate that nuclear localization of Chfr is required for p21 expression, which results in subsequent cell cycle arrest.

DISCUSSION

Previous studies have indicated that the FHA domain of the Chfr protein is essential for its localization in the nucleus in promyelocytic leukemia (PML) foci (Daniels et al., 2004). Since the ΔN195 mutant Chfr protein was localized in the nucleus, this shows that the FHA domain is important for Chfr subnuclear localization to PML bodies. On the other hand, recent study reported that endogenous highly overexpressed ectopic Chfr protein has different cell localization characteristics (Burgess et al., 2008). According to this report, endogenous and lowly expressed ectopic Chfr is cytoplasmic and localized to the spindle during mitosis, whereas highly expressed Chfr is associated with a shift in localization to the nucleus/PML bodies. We found that full-length Chfr has an NLS sequence that is essential for its nuclear localization. NLSm Chfr does not exist in the nucleus, although the expression levels of wild-type and NLSm Chfr were almost similar. These findings indicate that rather than being a simple consequence of Chfr over-expression, the NLS is responsible for nuclear localization of the Chfr protein, although the underlying mechanism remains to be elucidated. We noted that the expression levels of wild-type and NLSm Chfr were almost similar and that the expression of the I306A mutant Chfr (which lacks Ub-ligase activity) was even higher. These results suggest that the cellular level of Chfr might be controlled by autoubiquitination. This is also consistent with our findings showing no difference between wild-type and NLSm mutant Chfr in autoubiquitination activity and stability. In addition, we examined the biological significance of the NLS in Chfr by focusing on a nuclear target of Chfr. Recently, HDAC1 was identified as a novel substrate of the Chfr protein. HDAC1 is a key transcriptional regulator involved in diverse cellular processes. It was found that by functioning as an E3 ubiquitin ligase for polyubiquitination of HDAC1, Chfr promotes the degradation of HDAC1 (Oh et al., 2009). It is possible that these events occur in the nucleus at interphase. In our study, the NLSm Chfr protein was unable to upregulate p21 relative to wild-type Chfr, due to impaired downregulation of HDAC1. Therefore, our findings indicate that localization of Chfr within the cell nucleus is crucial to its function as a tumor suppressor and cell cycle checkpoint regulator. Taken together, our data highlight the importance of nuclear localization in mediating the physiological roles of Chfr.

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