Proteins interacting with CHFR, mitotic-checkpoint ubiquitin ligase

Hiroaki Mita[1], Minoru Toyota[2,3], Yasushi Sasaki[1], Hiromu Suzuki[4], Masashi Idogawa[1], Lisa Kashima[1] and Takashi Tokino[1]

[1]Department of Molecular Biology, Cancer Research Institute, Sapporo Medical University, Sapporo, Japan [2]First Department of Internal Medicine, Sapporo Medical University, Sapporo, Japan [3]Precursory Research for Embryonic Science and Technology; Japan Science and Technology Agency, Kawaguchi, Japan [4]Department of Public Health, Sapporo Medical University, Sapporo, Japan

ABSTRACT

Cell cycle progression is monitored by checkpoint mechanisms to ensure the integrity of the genome. CHFR which contains a RING domain and has ubiquitin ligase activity, a novel mitotic checkpoint gene, delays chromosome condensation in cells treated with microtubule poisons. CHFR is inactivated by promoter methylation and point mutations in various human tumors, and cancer cells lacking CHFR are sensitive to microtubule inhibitors. However, few reports are available on the molecular mechanism that accounts for the link between the sensitivity of cancer cells to microtubule inhibitors and the physiological function of CHFR. In the present study, we isolated cellular proteins capable of interacting with CHFR using yeast two-hybrid method to clarify the function of CHFR. As a result of the screening, we isolated canonical and noncanonical E2 ubiquitin conjugating enzymes as CHFR interacting proteins, which are involved in proteolytic and non-proteolytic ubiquitination respectively. This raises the possibility that CHFR is switching canonical and non-canonical ubiquitination depending on the situation of cells. On the other hand, we isolated gadd34 which interacted with the FHA domain of CHFR by two-hybrid screen. Coexpression in mammalian cells showed that gadd34 interacted with the FHA domain of CHFR, but gadd34 is not the substrate for CHFR, rather it promoted autoubiquitination of CHFR. Furthermore, CHFR moved, in part, from nucleus to cytoplasm in the presence of microtubule inhibitor docetaxel, which enabled colocalization of CHFR and gadd34 in cytoplasm. This colocalization was followed by cell death. These findings suggest that gadd34 and CHFR cooperate to mediate cell death in response to mitotic stress.

Key words : Ubiquitination, Microtubule inhibitor, Cell cycle

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CORRESPONDENCE TO : Takashi Tokino, Professor
Department of Molecular Biology, Cancer Research Institute,
Sapporo Medical University
South 1, West 17, Chuo-ku, Sapporo 060-8556, Japan.
E-mail : tokino@sapmed.ac.jp
TEL : 011-611-2111(ext 2386) FAX : 011-618-3313

INTRODUCTION

For normal cell cycle progression, precise regulation of DNA condensation and chromosome segregation in mitosis is important[1]. Since mitosis requires the ordered execution of several biochemically distinct processes, eukaryotic cells have developed checkpoint mechanisms to ensure the timely execution of these events. The most studied mitotic checkpoint is the spindle assembly checkpoint, which inhibits sister chromatid separation until all chromosomes have aligned on the mitotic spindle[2]. Although the integrity of the spindle checkpoint is essential for the maintenance of chromosomal stability, spindle checkpoint proteins and APC components are rarely downregulated or mutated in tumors[3]. Recent evidence also suggests the presence of another checkpoint in mammalian cells, upstream of the spindle checkpoint. This checkpoint delays chromosome condensation in response to mitotic stress and is regulated by the expression of a protein designated CHFR (checkpoint with FHA and RING finger) [4].

Although CHFR is ubiquitously expressed in normal tissues, it is frequently downregulated in human cancers, mostly owing to hypermethylation of its promoter region [5]. CHFR downregulation has been found in primary carcinomas and tumor cell lines of oral, esophageal, gastric, colon, lung, breast, brain, bone and hematopoietic origin[4–11]. Mutations in CHFR have also been identified in primary lung cancers and cell lines including osteosarcoma and breast cancer[4, 9, 10]. Importantly, the cells that did not express CHFR were especially sensitive to microtubule inhibitors, resulting from impaired checkpoint function in human cancer cell lines[5, 8, 12]. Thus, the loss of CHFR expression by aberrant methylation may possibly predict the responsiveness of human cancers to microtubule inhibitors, such as paclitaxel or docetaxel.

Although recent advances have led to a better understanding of the function of CHFR as a mitotic checkpoint gene, the results obtained so far are controversial. The protein encoded by CHFR contains FHA and RING domains and a cysteine-rich C-terminus. Crystallographic evidence suggests that the FHA domain of CHFR binds phosphorylated proteins[13], as previously reported for other FHA domains[14]. The FHA domain of CHFR is essential for invoking mitotic checkpoint. Some colon carcinoma cell lines express CHFR lacking the FHA domain (CHFR-ΔFHA) that acts as dominant negative form[4, 5]. Despite the importance of the FHA domain, proteins binding to the FHA domain in CHFR have not been clarified.

The RING domain of CHFR is necessary to interact with E2 ubiquitin conjugating enzymes and has E3 ubiquitin ligase activity in vitro[12, 15, 16], consistent with previous analyses of other proteins with RING domains[17]. One potential CHFR substrate is polo-like kinase 1 (PLK1) [15], which phospyorylates cyclin B1 in mid prophase and induces the nuclear localization of cyclin B1 required for chromosome condensation[18, 19]. In fact, CHFR delays chromosome condensation and is involved in inhibition of accumulation of cyclin B1 in the nucleus[8, 20]. However, the expression of PLK1 does not always correlate with CHFR downregulation and CHFR function in breast cancer cell lines[10]. Furthermore, no degradation of PLK1 was observed in CHFR expressing cells even after the checkpoint was activated by microtubule inhibitor nocodazol or corcemid[20, 21]. Another potential CHFR substrate is Aurora A[22], which is required for centrosome maturation, spindle assembly, and also regulates the intracellular localization of cyclin B1[23]. Aurora A is frequently upregulated in variety of tumors [24, 25]. Yu et al. showed that CHFR interacted with, ubiquitinated and then induced degradation of Aurora A in vitro and vivo[22]. Furthermore, embryonic fibroblasts of CHFR knockout mouse upregulated expression of Aurora A, showed chromosomal instability and developed spontaneous tumors, suggesting that CHFR is a tumor suppressor[22]. In contrast, the analysis of HCT116 colon carcinoma cells stably expressing CHFR indicated that there was no difference in Aurora A protein levels as a function of CHFR expression[20]. In addition, CHFR in-

activation was not associated with chromosomal instability in colon cancers[26]. A Similar dispute remains regarding whether autoubiquitination of CHFR plays a role in degradation of CHFR itself or not[12, 16]. More Recently, Bothos et al. reported that CHFR functions with UBE2N-Mms 2, a heterodimer of E2 ubiquitin conjugating enzymes to catalyze noncanonical Lys63-linked polyubiquitination[16]. Compared with the canonical Lys48-ubiquitination, Lys63-linked polyubiquitination is not associated with degradation of target proteins, but rather with signaling cellular stress independent of degradation[27-29]. To date, the noncanonical ubiquitination by CHFR has been observed only in the autoubiquitination of CHFR[16]. These findings disclosed complicated roles of the RING domain in CHFR. By contrast, few reports are available on the molecular mechanism that explains the link between sensitivity of cancer cells to microtubule inhibitor and the function of CHFR.

To explore the physiological function of CHFR and its role in response to microtubule inhibitor, we comprehensively investigated CHFR-interacting proteins by yeast two-hybrid screen using CHFR as bait. We isolated noncanonical E2 UBE2N besides canonical E2 enzymes, and confirmed that the stability of CHFR is retained though the cell cycle, even in the presence of microtubule inhibitor. Furthermore, we found gadd34 as a novel CHFR-FHA binding protein that induced autoubiquitination of CHFR. Colocalization of CHFR and gadd34 was observed when the cells were exposed to the microtubule inhibitor, and cell death resulted. These findings suggest that gadd34 binds and regulates ubiquitin ligase activity of CHFR, and they cooperate to mediate cell death by microtubule inhibitor.

MATERIALS AND METHODS

Yeast and bacterial strains

The yeast strain, L40 (MATa leu2 trp1 his3 ade2 LYS2::lexAop-HIS3 URA3::lexAop-lacZ gal 80) was used for two-hybrid screening and interaction analyses. E.coli DH10B (F-, mcrA, Δ (mrr-hsdRMS-mcrBC), Φ80dlacZ, DM15, DlacX 74, deoR, recA1, araD139, Δ(ara leu)7697, galU, galK, λ-, rpsL, endA1,nupG) (Takara) was used for plasmid construction. E.coli HB101 (supE44, D(mcrC-mrr), recA13, ara-14, proA2, lacY1, galK2, rpsL20, xyl-5, mtl-1, leuB6, thi-1) (Clontech) was used to recover expression plasmids from yeast.

Buffers and media

Lysis buffer contained sodium-phosphate (10 mM Tris, pH 7.4) containing 150 mM NaCl, 10mM CHAPS, 1mM NaF, 1mM sodium vanadate and a protease inhibitor cocktail (Roche). Sodium dodecyl sulfate (SDS) loading buffer is 65 mM Tris-HCl (pH 6.8), 5% (vol/vol) 2-mercaptoethanol, 2% SDS, 0.1% bromophenol blue and 10% glycerol. The Yeast strain L40 was grown in media as follows: Yeast extract-peptone-dextrose (YPD) medium containing 10 g of yeast extract, 20 g of tryptone, and 20 g of dextrose per liter; SD (2% glucose, 0.67% yeast nitrogen base without amino acids) with appropriate supplements; CAD-W, SD with 0.5% casamino acids.

Yeast expression plasmids

The complete open reading frames (ORFs) of human CHFR (Accession: BC012072, DB-CHFR-Full, amino acids (aa) 1-652), CHFR lacking the FHA domain (DB-CHFR-DFHA, aa 147-652) and the N-terminus of CHFR containing the FHA domain (DB-CHFR-FHA, aa 1-186) were individually cloned into pBTM118[30] for LexA DNA-binding domain (DB) fusion proteins (Fig. 1A). DB-MKK6 was described previously[30].

Isolation of CHFR-interacting molecules by yeast two-hybrid screening

Yeast L40 cells were transformed with either construct of DB-CHFR-Full, -ΔFHA or -FHA and grown on CAD-W plates. Each single colony of L40 transformed with one of DB-CHFRs was expanded in CAD-W media, and then the cells were transformed with a human placenta cDNA library (Clontech) in the pACTII

plasmid for GAL4 activator domain (ACT) fusion proteins. Aliquots were taken from each transformation mix before plating and used to determine the transformation efficiency by plating on SD media lacking tryptophan and leucine. The transformants were plated onto SD media lacking tryptophan, leucine and histidine but including 20mM 3-AT (Sigma) and incubated at 30℃ for 4 - 14 days. The His+ colonies were picked up and were grown in SD lacking leucine to enable segregation of DB-CHFRs. The pACTII library plasmids were recovered by a PI-100 automatic plasmid isolation system (Kurabo) using the protocol for yeast plasmid isolation, and transformed into HB101 E. coli strain on an M9 plate containing ampicillin but lacking leucine. Each pACTII library plasmid recovered from HB101 and DB-CHFR was re-transformed into L40, and each colony was streaked on both a selective medium lacking leucine, tryptophan, and a medium which also lacked histidine to verify histidine prototrophy.

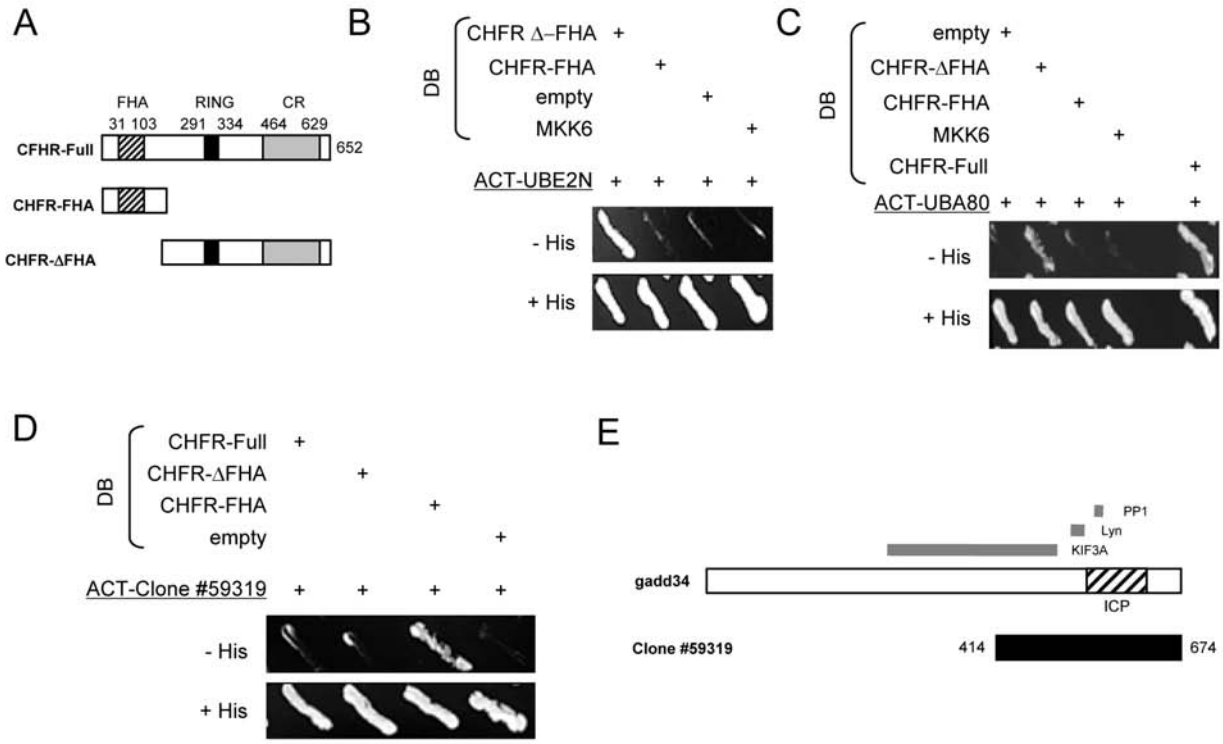

Fig.1 Isolation of CHFR interacting molecules. **A.** The structural domains of CHFR used in BD constructs for yeast two-hybrid screening. FHA, forkhead-associated domain; RING, ring finger domain; CR, cysteine-rich region. The numbers refer to amino-acid positions. **B and C.** Interaction between CHFR and ubiquitin related molecules. Yeast L40 cells were transformed with two plasmids (either ACT-UBE2N or ACT-UBA80, and DB-CHFR constructs) as indicated. A plus sign indicates the vectors used for the interaction analysis. DB-MKK6 was used as a negative control. Transformed cells were spread on the appropriate synthetic agar plates supplemented with (+His) or without (-His) histidine. **D.** Interaction between CHFR and isolated cDNA clone #59319. **E.** The domain map of full-length human gadd34 protein. The herpes simplex virus ICP34.5 homology domain (ICP) [32] is shown by striped box. Above the domain map, regions reported to be required for the interactions with other proteins [32-34] were indicated by gray bars. Below the map, the region (amino acids 414 - 674) corresponding with the partial gadd34 cDNA contained in the positive clone #59319 is indicated by a black bar. The numbers refer to amino-acid positions.

For each two-hybrid test, LacZ activity was also visualized in situ by using 5-bromo-4-chloro-3-indolyl-s-D-galactopyranoside (X-Gal) as previously described [30]. Finally, the pACTII library inserts were sequenced by dye terminator cycle sequencing with ABI 3100 and BigDye Terminator Version 3.1 (Applied Biosystems).

Cell lines and reagents

Hela, Cos-7, two colorectal cancer cell lines (HCT116 and SW480), one gastric cancer cell line (SNU1), one oral cancer cell line (HSC3) and one osteosarcoma cell line (Saos2) were obtained from the American Type Culture Collection or Japanese Collection of Research Bioresources. Hela and Cos-7 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin, and streptomycin. Cells grown in 35-mm-diameter dishes were transfected with the appropriate expression plasmids with Lipofectamine 2000 reagent (Invitrogen) and treated by 1 mM docetaxel (Aventis) or 50 mM MG132 (Pepetide Institute) for the indicated time. The total amount of plasmid DNA was adjusted to either 2 mg per plate with empty vector DNA. Thirty-six to forty eight hours after transfection, cells were lysed in lysis buffer.

Mammalian expression plasmids

Flag-tagged full length CHFR and its deletion mutants, that is, CHFR-DFHA, CFHR-FHA, CHFR lacking the RING domain (CHFR-DRING, Δ aa 291-334) and the C-terminus of CHFR containing the cysteine-rich domain (CHFR-CR, aa 418-652) were generated by PCR -based method, using pCMV-tag2B or -tag2C (Stratagene) as a backbone. GFP-CHFR was generated by subcloning full length of CHFR into pEGFP-C1 (Clontech) vector. The complete open reading frames of human gadd34 (Accession: FLJ10499), provided by National Institute of Technology and Evaluation, Biological Resource Center, Japan, were subcloned into pCMV-tag3B (Stratagene), pEGFP-C1 and pDsRed2-N1 (Clontech) vectors to generate mammalian expression vectors of myc-tagged gadd34, EGFP-gadd34 and gadd34-DsRed2, respectively.

Stable transfection of cell lines

Hela cells were transfected with 2 mg of plasmid, pEGFP-CHFR-Full or pEGFP. Forty-eight hours later, cells were trypsinized, counted, and plated in 100-mm-diameter dishes at three different cell densities (10,000, 1,000, and 100 cells/plate). Twenty-four hours later, neomycin (G418; Geneticin; Gibco BRL), at 500 mg/ml, was added to the medium and the cells were incubated in the presence of the drug (replaced every 4 days) for a total of 2 weeks. Individual clones (<200 cells/clone) were isolated with Pyrex cloning rings, trypsinized, and added to 24-well plates. The isolated clones were expanded, and aliquots were frozen at -80℃. All subsequent culture of the clones was performed in the presence of neomycin (100 mg/ml) to minimize the expansion of revertant cells.

Immunoblot analysis

Cell lysates were separated by 10% SDS-PAGE, transferred to an Immobilon-P membrane (Millipore), probed with each antibody and visualized using enhanced chemiluminescence (Amersham). The following antibodies were used: mouse monoclonal antibody (mAb) M2 specific to the Flag epitope (Sigma); mouse mAb 9E10 specific to the myc epitope (Santa Cruz); mouse mAb specific to EGFP (Santa Cruz); and mouse mAb specific to ubiquitin (Santa Cruz).

Coimmunoprecipitation assay

Immunocomplexes were recovered from cell lysates with the aid of anti-Flag M2 agarose affinity gel (Sigma) or agarose conjugated anti-myc-tag (MBL), washed three times with the lysis buffer. Immunoprecipitates were resuspended in 30 ul of SDS loading buffer, boiled for 5 min and separated by SDS-PAGE.

Immunofluorescent staining

To immunofluorescently stain cells, cells fixed in 3.7% formalin solution for 10 min at 25℃ were washed with PBS and then incubated overnight with rabbit anti-Flag or anti-myc mAb. FITC-conjugated goat anti-mouse Ab (Molecular Probes) was used as a secondary antibody. Nuclei were visualized using DAPI. Cells were examined with a confocal microscope (FV 300, Olympus).

Flow cytometry analysis

Cells were treated with 1 mM docetaxel or 50mM MG132 and then harvested. The Cells were fixed in 10% paraformaldehyde for 10 min, then fixed in 70% ethanol for 30 min, incubated with 2 mg/ml RNase, and stained in 50 mg/ml of propidium iodide solution. Approximately 5x 10^4 stained cells were analyzed using a FACScan flow cytometer (Becton Dickinson).

Time lapse imaging

Hela or Cos-7 cells which transiently or stably expressed either or both of GFP-CHFR and DsRed2-gadd34 were grown on a 35mm glass base dish (IWAKI). Cells were placed in a CO_2 chamber with heater attached on the microscope stage, and the time lapse imaging of GFP-CHFR and DsRed2-gadd34 was performed using a confocal laser scanning microscope (FV 300, IX71-BG-SP system, Olympus). Images were captured and analyzed by Fluoview software (Olympus).

Quantitative RT-PCR

Single-stranded cDNA was prepared using Superscript III reverse transcriptase (Invitrogen). Real-time PCR was carried out using an ABI Prism 7000 Sequence Detection System (Applied Biosystems) in a solution containing 1x Taqman Universal PCR Mater Mix (Applied Biosystems), 1 μL cDNA and 0.5 mmol/L of each primer. Levels of each gene expression were normalized to the signal from glyceraldehydes-3-phosphate dehydrogenase (GAPDH). PCR protocol was as follows: 95℃, 1 minute, 40 cycles of 30 seconds at 95℃, 1 minute at 60℃. The mixtures of primers and taqman probes purchased as Gene Expression Assay (GEA) (Applied Biosystems) were as follows: Hs 00169585_m1 for gadd34; Hs00217191_m1 for CHFR.

RESULTS

Isolation of CHFR-Binding Proteins

In order to identify human proteins that interact with the CHFR E3 ubiquitin ligase, a human placenta cDNA library constructed with a GAL4-activator-domain (ACT) vector was screened by a yeast two-hybrid method. The bait used was a fusion construct between the LexA DNA-binding-domain (DB) and full-length CHFR (CHFR-full) (Fig. 1A). In addition, the FHA domain of CHFR (CHFR-FHA) or CHFR lacking the FHA domain (CHFR-ΔFHA) were also constructed as bait because FHA domain is generally required for the interaction with phosphorylated proteins and some cancer cells express FHA-deleted form of CHFR, which suggests the importance of the FHA domain in tumorigenesis.

A total of ～ 2 million transformants were placed under selection (Table 1). To test further whether the phenotype observed in the original screen was reproducible and dependent on the CHFR hybrid, library-derived plasmids were selectively recovered from the transformants spread on synthetic complete -His, Leu, Trp

Table 1 Summary of the yeast two hybrid screening and rcscreening results

	Bait CHFR			Total
	Full	ΔFHA	FHA	
#of transformants	1,161,000	386,600	457,600	2,005,200
#of His+	113	59	159	331
#of CHFR-dependent His+	57	19	19	95

plus 20mM 3-AT plates, by virtue of the yeast LEU2 gene carried on those plasmids to complement a leuB6 mutation present in the E. coli strain HB101. Each isolated plasmids was then used to transform L40 with either of the CHFR baits. Transformants were assayed again for histidine prototrophy and beta-gal activity, and those showing activity only in the presence of bait CHFR were considered positive (Table 1). Of the 331 original isolates, 95 of the recovered plasmids induced the expression of histidine and did so only in the presence of bait CHFR.

Positive clones encode ubiquitin related proteins

Sequence analysis of the positive clones revealed that 12 out of 95 cDNA inserts encoded full length of E2 ubiquitin conjugating enzymes, UBE2N, UBE2E3, UBE2D2 and UBE2D3 (Table 2). CHFR is a RING-type E3 ubiquitin ligase, which interacts with E2 enzymes via its RING domain and induces ubiquitination of its substrate proteins 15, 22). Isolation of E2 enzymes as a CHFR interactor proved that the screening worked well. Consistently, no E2 enzyme was isolated when DB-CHFR-FHA was used as bait because of the lack of the RING domain that is essential for the direct interaction with E2 enzymes (Table 2).

It is noteworthy that noncanonical E2 enzyme UBE2N was isolated, which induces non-proteolytic K63-linked polyubiquitination of target proteins. Two-hybrid analysis showed that the interaction between CHFR and UBE2N was independent of the FHA domain, and probably dependent on the presence of the RING domain (Fig. 1B). This finding supports other reports that CHFR is involved in non-proteolytic ubiquitination.

On the other hand, 41 out of 95 cDNA inserts encoded ubiquitin precursor proteins, that is, UBA80, UBA52, ubiquitin B and ubiquitin C (Table 2). Ubiquitin is synthesized as precursor proteins that consist either of tandem ubiquitin chains that are cleaved into moieties of the ubiquitin B or ubiquitin C types, or single ubiquitin moieties fused 5-prime to unrelated carboxyl extension proteins (UBA type) such as UBA80 and UBA52. Ubiquitin sequences are highly conserved from species ranging from yeast to human, and the ubiquitin precursors are rapidly processed to free ubiquitin monomer even when artificially overexpressed in yeast [31]. Considering that CHFR is reportedly autoubiquitinated, the ACT-fused ubiquitin precursors were processed as ubiquitin monomer in yeast, and could have resulted in covalent extension of CHFR, rather than protein-protein in-

Table 2 Summary of CHFR interacting molecules isolated by the yeast two hybrid screening

		#of CHFR-dependent His+			
		Bait CHFR			Total
Function	cDNA	Full	ΔFHA	FHA	
Ubiquitin precursor	UBA80	18	4	0	22
	UBA52	12	1	0	13
	Ubiquitin B	4	1	0	5
	Ubiquitin C	1	0	0	1
E2 enzyme	UBE2N	3	2	0	5
	UBE2E3	2	1	0	3
	UBE2D2	1	2	0	3
	UBE2D3	1	0	0	1
Linker of ubiquitination mechinery	Ubiquilin 1	1	0	0	1
Pratein Phosphatase 1 regulatory subunit	gadd34	0	0	1	1
Others		14	8	18	40
	Total	57	19	19	95

teraction with CHFR. Supporting this notion, no ubiquitin precursor was isolated by DB-CHFR-FHA that had no ubiquitin ligase activity because of lack of the RING domain (Table 2). Furthermore, two-hybrid analysis showed the positive results between ACT-UBA80 and both of BD-CHFR-full and BD-CHFR-ΔFHA, but not BD-CHFR-FHA (Fig. 1C). These suggest that the processed ACT-ubiquitin was bound to CHFR by RING-dependent autoubiquitination.

Identification of gadd34 as an interactor with CHFR

Differently from ubiquitin related molecules, some clones showed positive interaction with the FHA domain of CHFR (Table 2). One of those clones, #59319, showed strong histidine prototrophy with BD-CHFR-FHA (Fig. 1D). Sequence analysis unveiled that the #59319 encodes the C-terminus (amino acids 414-674) of gadd34 (Fig. 1E), which was originally identified as a gene transiently expressed in growth-arrested cells with DNA damage and characterized later as a protein phosphatase 1 (PP1) regulatory subunit [32]. The isolated C-terminus of gadd34 contained the region which is reported to be required for the interaction with various other proteins, that is, PP1[32], Lyn[33] and KIF3A[34].

Interaction of gadd34 with CHFR in mammalian cells was investigated using in vivo coprecipitation experiments. To determine the specific domain in CHFR that is essential for binding gadd34, various Flag-tagged segments of CHFR (Fig. 2A) and the myc-tagged full-length gadd34 were cotransfected into Hela cells. Flag-tagged proteins were immunoprecipitated, and coprecipitated myc-gadd34 was probed by an anti-myc monoclonal antibody. As shown in Figure 2C, both full-length CHFR and CHFR-FHA coprecipitated gadd34, whereas DFHA did not. CHFR-ΔRING coprecipitated gadd34 much more than the full-length CHFR. The treatment by microtubule inhibitor docetaxel slightly increased gadd34 coprecipitation by full-length CHFR. These results demonstrated that interaction of CHFR and gadd34 protein can occur in mammalian cells, and the FHA, but not the RING domain of CHFR is required for binding to gadd34.

gadd34 regulates the activity of E3 ubiquitin ligase, CHFR

To clarify the physiological meanings of the interaction between CHFR and gadd34, we assessed the ubiquitination and protein stability of gadd34, and investigated the possibility that gadd34 is a substrate for ubiquitination by CHFR. Western blot analysis of total lysate (Fig. 2B) showed that the stability of myc-gadd34 was almost unaffected by coexpression of CHFR or docetaxel treatment. Furthermore, high-molecular-weight ubiquitinated proteins were immunoprecipitated with transfected Flag-CHFR-ΔFHA although gadd34 did not interact with CHFR-ΔFHA at all (Fig. 2C, Fig 1D). These findings indicated that it was unlikely that gadd 34 was the substrate for ubiquitination by CHFR.

On the other hand, high-molecular-weight ubiquitinated proteins were observed in immunoprecipitates with Flag-CHFR-Full and Flag-CHFR-ΔFHA that retain the RING domains, which were essential for ubiquitin ligase activity. The ubiquitinated proteins were decreased in the immunoprecipitates with transfected Flag-CHFR-ΔRING, or dismissed in that with Flag-CHFR-CR (Fig. 2C). Considering the above finding that Flag-CHFR-ΔFHA did not interact with myc-gadd34 but that the immunoprecipitates showed ubiquitination (Fig. 2C, lane 5), detected ubiquitinated proteins were CHFR themselves due to autoubiquitination. Interestingly, autoubiquitination of Flag-CHFR was much increased when myc-gadd34 was cotransfected (Fig. 2C, lane3) in comparison with when no myc-gadd34 was (Fig. 2C, lane7). These suggest that gadd34 is a positive regulator of ubiquitin ligase CHFR, but not a substrate for CHFR.

Stability of CHFR though the cell cycle

Although gadd34 seemed to induce the aut-

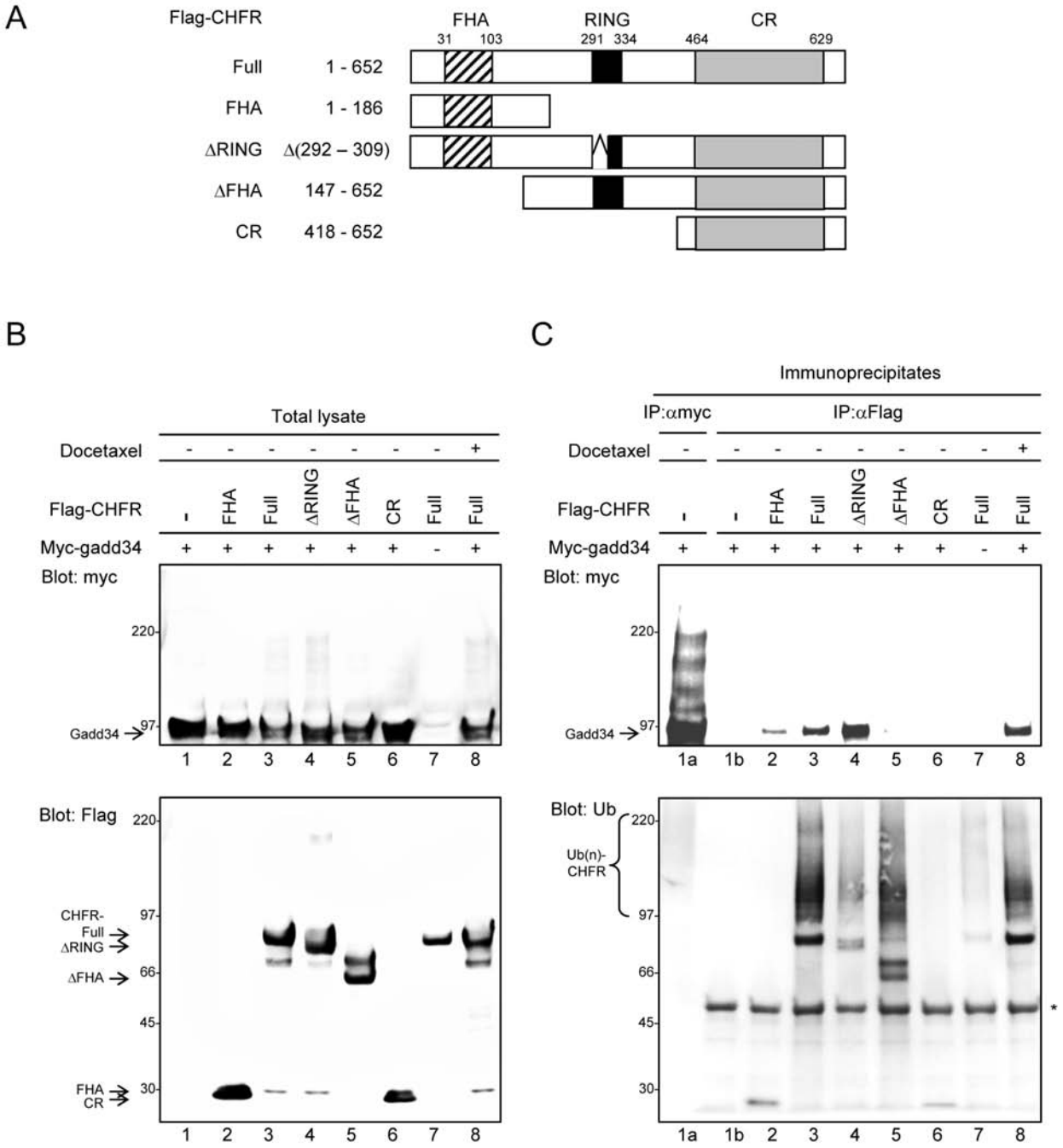

Fig.2 gadd34 was interacted with CHFR in mammalian cells and regulated ubiquitin ligase activity of CHFR. **A.** The scheme of Flag-CHFR constructs used for coimmunoprecipitation assay. **B and C**. Coimmunoprecipitation assay between Flag-CHFR and myc-gadd34. Hela cells were cotransfected with Flag-CHFR-Full or its deletion mutants together with myc-tagged full-length gadd34. Expression of Flag-CHFR and myc-gadd34 was examined using total lysates (B). Immunoprecipitates with anti-Flag antibody were blotted by anti-myc antibody (C, the upper blot) or by anti-ubiquitin antibody (C, the lower blot). The total lysate of Hela cells transfected with myc-gadd34 alone (B, lane #1) was precipitated by anti-myc antibody (C, lane #1a) or by anti-Flag antibody (C, lane #1b), as controls. Only the sample loaded in lane # 8 was extracted from the cells coexpressing Flag-CHFR and myc-gadd34 which were treated by 1 mM docetaxel for 12 hr.

oubiquitination of CHFR, it was not clear that whether CHFR was degraded via canonical K48 -linked autoubiquitination or was involved in other signaling events via noncanonical K63-linked autoubiquitination, which did not induce degradation of CHFR. To address this question, we sought to observe the stability of CHFR through the cell cycle in the presence or absence of mitotic stress, because CHFR is a mitotic check point gene and is involved in cell death by microtubule inhibitors. We first established Hela cells which stably express GFP-fused CHFR (Hela D2) or GFP alone (Hela F22) as a control. The GFP was used as a reporter to examine stability of each protein. Fluorescent microscopic analysis of living cells showed that GFP-CHFR was localized in nuclei of Hela D2 cells (Fig. 3A), and its nuclear localization was consistent with that of transiently expressed Flag-CHFR-Full (Fig. 3B). Treatment by the microtubule inhibitor docetaxel did not change the signal intensity or localization of the GFP-CHFR in Hela D2 (Fig. 3A). On the other hand, treatment by the proteasome inhibitor MG132 that blocks degradation of ubiquitinated proteins increased stability of GFP-CHFR much more than GFP (Fig. 3, A and C). These suggest that CHFR is usually degraded at a constant rate in cells.

To investigate if changes in the stability of CHFR are dependent on the cell cycle stage in each cell, we next analyzed simultaneously both of CHFR stability and cell cycle by flow cytometry (Fig. 4). GFP intensity, which represents the amount of GFP protein in each cell, of asynchronous Hela D2 was not changed through the cell

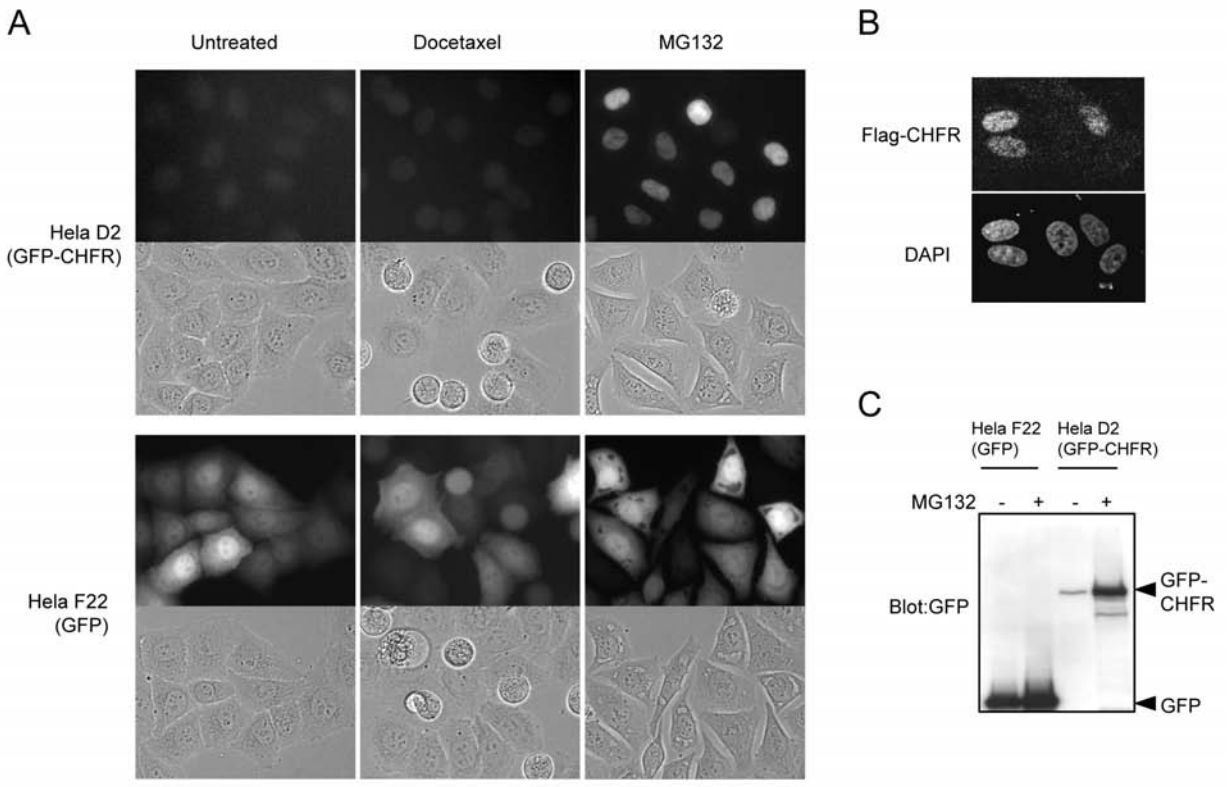

Fig.3 Establishment of Hela cells which stably express GFP-CHFR. **A.** Expression and subcellular localization of GFP-CHFR in G418 selected Hela cells. Microscopic analyses of living cells were performed for cells untreated, treated with docetaxel (1 μM for 16 hr) or MG132 (50 μM for 12 hr) by combinations of fluorescent and phase contrast imaging. **B.** Immunofluorescent staining of transiently transfected Flag-CHFR in Hela cells. Nuclei were stained by DAPI. **C.** Immunoblot analysis of GFP-CHFR stably expressed in Hela cells with or without MG132 treatment for 12 hr.

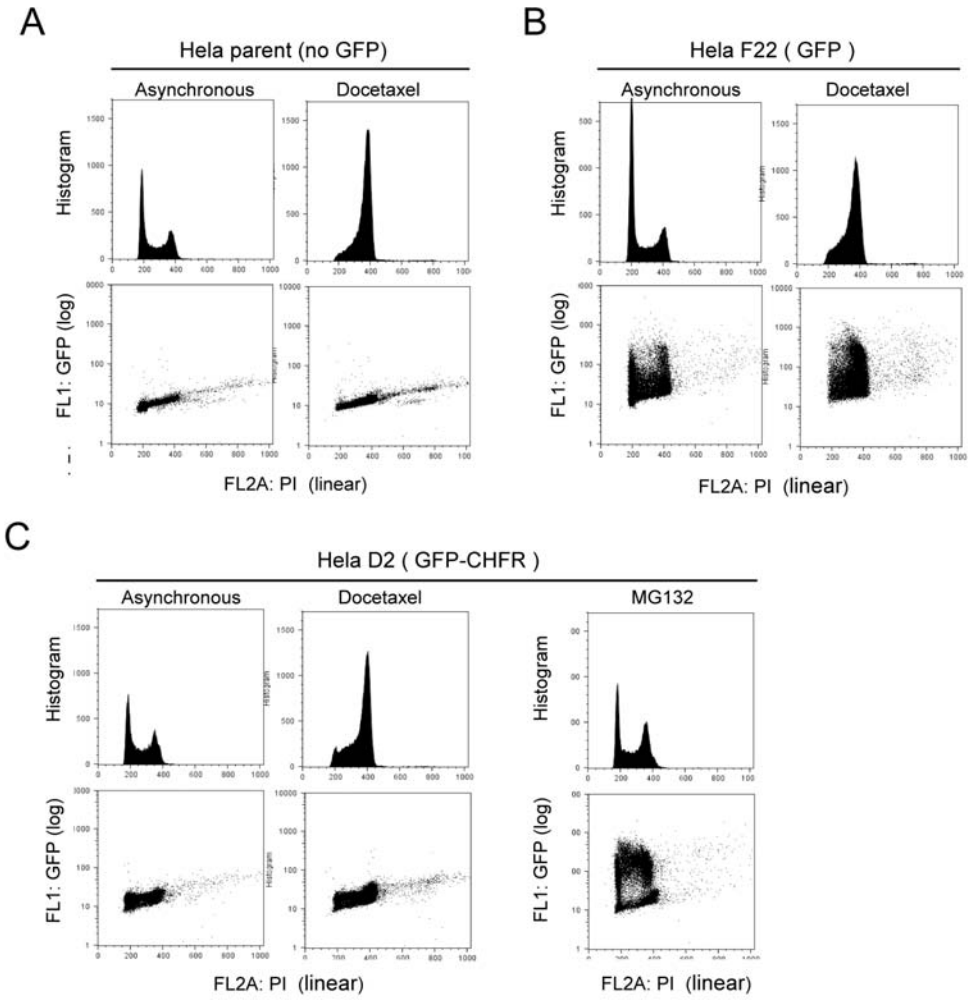

Fig.4 Stability of GFP-CHFR in Hela cells under mitotic stress. Hela parent cells with no GFP (A), Hela F22 cells which stably express GFP (B) and Hela D2 cells which stably express GFP-CHFR (C) in the presence (1 μM for 16 hr) or absence of microtubule inhibitor docetaxel, were subjected to FACS analysis. X axis means DNA content determined by propidium iodide (PI) staining in all histograms and plots. Each upper histogram is the result of usual cell cycle analysis by PI staining, in which Y axis means the number of cells. In each lower dot plot, fluorescent intensity of GFP, which represents protein amount of GFP or GFP-CHFR in each cell, is shown in Y axis. As a control, Hela D2 cells were treated by MG132 (50 μM for 12 hr) (C).

cycle (Fig. 4C). After the mitotic stress induced by docetaxel treatment, all of Hela-parent, -F22 (GFP) and -D2 (GFP-CHFR) cells showed accumulation in G2/M phase (Fig. 4, ABC). The amount of GFP-CHFR in Hela D2 cells which were accumulated in G2/M was not changed whereas the Hela D2 cells treated by the proteasome inhibitor MG132 showed upregulation of GFP-CHFR through the cell cycle (Fig. 4C). These suggest that CHFR functions as a mitotic checkpoint gene and is involved in response to microtubule inhibitors via mechanisms other than cell-cycle- or mitotic-stress-dependent stabilization of CHFR.

Gadd34 was localized in the cytoplasm

We next examined if mitotic stress affects gadd34 behavior in cells or not. Fluorescent immunostaining analysis of myc-gadd34 (Fig. 5A), which was transiently transfected in Hela cells, revealed cytoplasmic localization of gadd34 in untreated cells. Docetaxel treatment did not

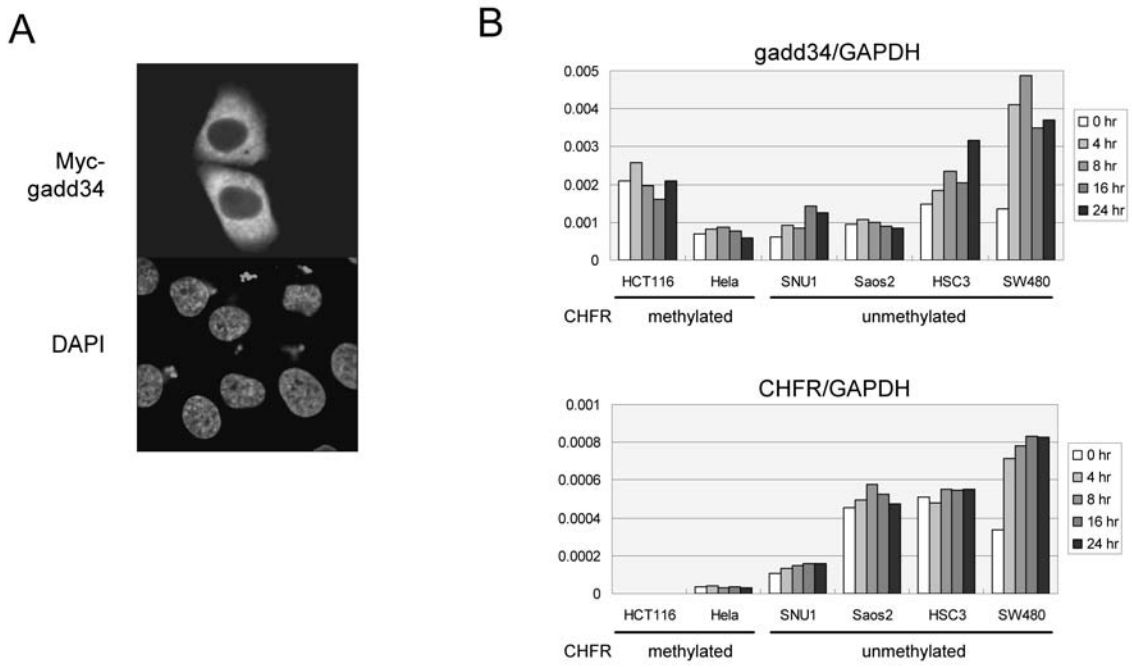

Fig.5 Expression and subcellular localization of gadd34 in Hela cells. **A.** Hela cells were transiently transfected with myc-gadd34 and immunostained by anti- myc antibody. **B.** mRNA expression of endogenous gadd34 and CHFR were examined by real time RT-PCR in various cancer cell lines exposed to 1 μM docetaxel. mRNA Expression of gadd34 and CHFR were corrected by dividing them with the expression of GAPDH in each cell line. Methylation status of CHFR promoter in each cell line is indicated below each graph.

change the cytoplasmic localization of myc-gadd34 (data not shown). We next analyzed mRNA expression of endogenous gadd34 in various cancer cells with or without aberrant promoter methylation of CHFR after the docetaxel treatment. Chronological expression analysis by real time RT-PCR revealed that mRNA of endogenous gadd34 did not show a time dependent increase over 24 hr after the docetaxel treatment, except for HSC3 and SW 480 cells. Furthermore, the basal expression level of gadd34 was independent on the expression of endogenous CHFR (Fig. 5B). These lead us to conjecture that interaction between gadd34 and CHFR is caused by change in their subcellular localization under mitotic stress, but not by transcriptional induction of gadd34 and CHFR.

Interaction between CHFR and gadd34 is caused by change in subcellular localization after the mitotic stress, and results in cell death.

We observed that CHFR and gadd34 were localized in the nucleus and cytoplasm respectively, which indicated mutually exclusive distribution in cells under the normal culture condition. In order to reveal the situation when and where CHFR interacts with gadd34 in cells, we chronologically observed the localization of each fluorescent-protein-fused CHFR and gadd34 in living cells by time-lapse confocal microscopy. Hela cells transiently transfected with GFP-CHFR alone (Fig. 6A) showed nuclear localization of GFP-CHFR before the metaphase and after the cytokinesis while GFP-CHFR was distributed in the whole area of cells except for the condensed chromosomal regions in the mitotic phase, that is, the metaphase and the telophase. Similar to GFP-CHFR, GFP-gadd34 was also

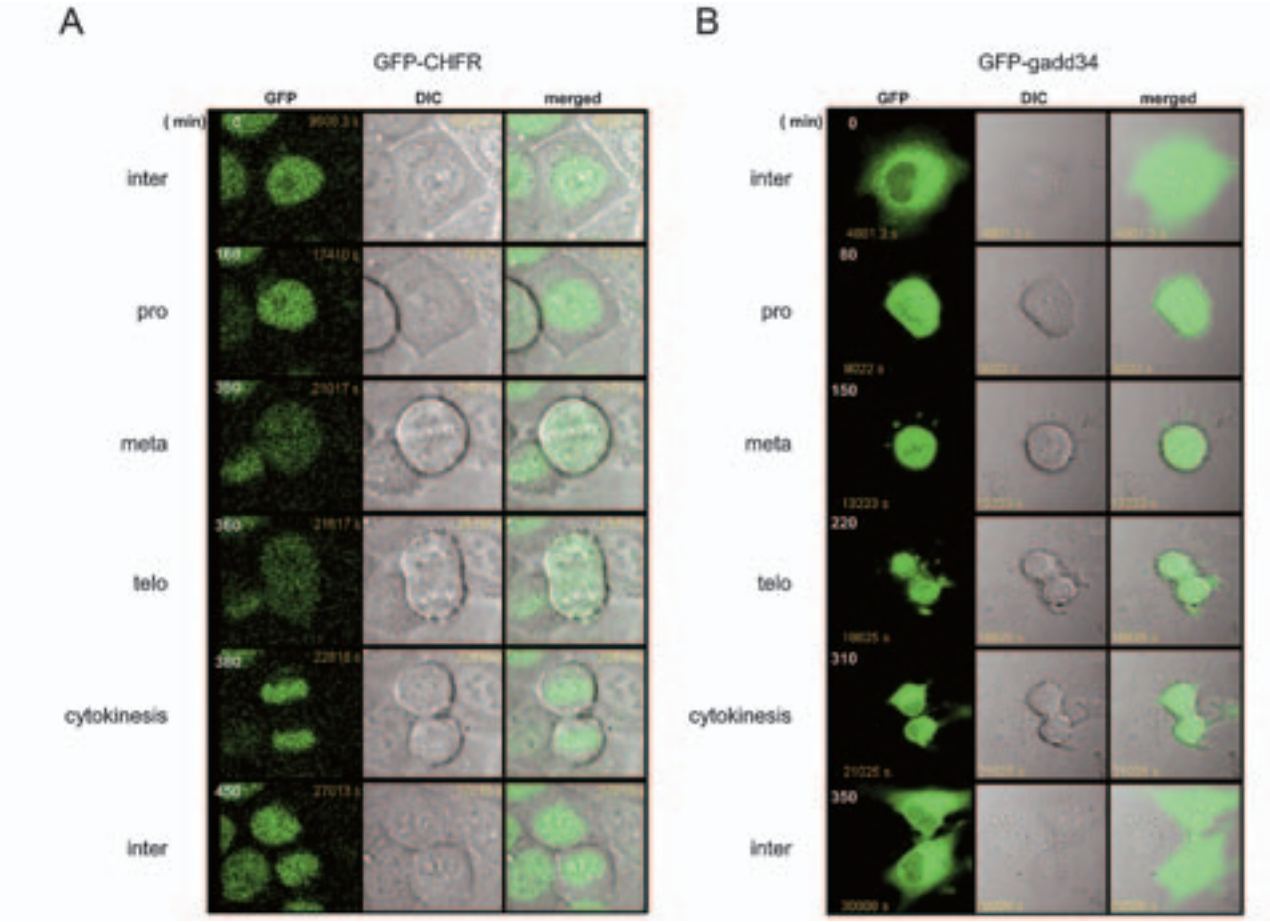

Fig.6 Time lapse analyses of subcellular localization of GFP-CHFR and GFP-gadd34. GFP-CHFR (A) and GFP-gadd34 (B) were transiently transfected into Hela and Cos7 cells respectively. Fluorescent and differential interference contrast (DIC) observations of living cells at indicated time points are shown. The stage of cell cycle in each cell is indicated left.

distributed in the whole area of mitotic cells except for chromosomal regions while the cytoplasmic localization of GFP-gadd34 was retained in the interphase (Fig. 6B). These findings suggest that CHFR interacts with gadd34 in the mitotic phase under normal culture condition. On the other hand, cotransfection of Hela cells with GFP-CHFR and DsRed-gadd34 showed that CHFR was recruited to the cytoplasm and partially colocalized with DsRed-gadd34 in the presence of docetaxel. Surprisingly, cells that showed the recruitment of GFP-CHFR to the cytoplasm were led to be ruptured and cell death in about 6 hr after the cytoplasmic recruitment of CHFR (Fig. 7). These suggest that recruitment of CHFR to the cytoplasm under mitotic stress induces the interaction with gadd34 and the consequential cell death.

DISCUSSION

In this paper, we isolated cellular proteins capable of interacting with CHFR using yeast two-hybrid method to clarify the function of CHFR. As a result of the screening, we isolated canonical and noncanonical E2 ubiquitin conjugating enzymes as CHFR interacting proteins. The result raised the possibility that CHFR switches canonical and noncanonical ubiquitination depending on the situation of cells, although how these two types of ubiquitination are chosen remains to be resolved. On the other hand, we isolated gadd34 which interacts with the

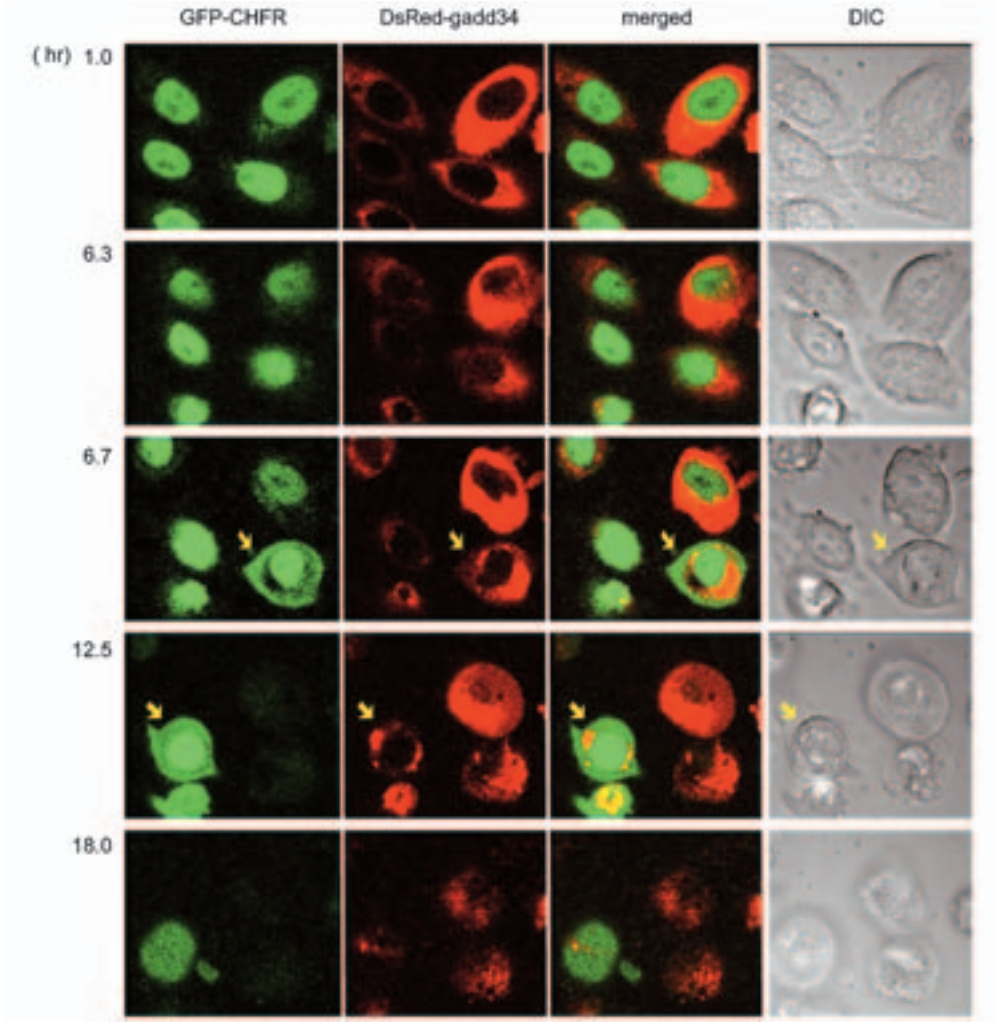

Fig.7 Colocalization CHFR and gadd34 observed before cell death induced by docetaxel. Hela cells were transiently cotransfected with GFP–CHFR and DsRed2–gadd34 and subjected to confocal time lapse imaging analysis after the addition of docetaxel. Fluorescent and differential interference contrast (DIC) observations of living cells at indicated time points are shown. The treatment by docetaxel (1 mM) was started at time = 0 hr. The cells, which showed cytoplasmic localization of GFP–CHFR and the subsequent cell death, are indicated by yellow allows.

FHA domain of CHFR. To our knowledge, this is the first report describing the protein which interacts with the FHA domain of CHFR. The biochemical analysis showed that gadd34 was not the substrate for CHFR, but rather induced autoubiquitination of CHFR. Furthermore, CHFR moved in part from nucleus to cytoplasm in the presence of microtubule inhibitor docetaxel, which enabled colocalization of CHFR and gadd34 in cytoplasm. This colocalization was linked to cell death. These suggest that gadd34 and CHFR cooperate to mediate cell death under mitotic stress.

Studies with our screening led to identification of four E2 conjugating enzymes including a noncanonical E2 enzyme, UBE2N, as CHFR interacting proteins. This finding does not conflict with either of the proteolytic ubiquitination of PLK1 or Aurora A being a key event caused by CHFR [15, 22] or with the fact that the non-proteolytic ubiquitination of CHFR itself is involved in the response to mitotic stress [16]. Our analysis

also showed that the stability of CHFR was not changed through whole stages of cell cycle. This result supports the Bothos's report [16] stating that CHFR was not degraded by proteolytic ubiquitination in a cell-cycle dependent manner. Inversely, we had also supposed that CHFR was stabilized when the cells were exposed to mitotic stress so that upregulated CHFR protein could invoke mitotic checkpoint to delay the chromosomal condensation. However, the protein level of CHFR was not increased even when the cells were exposed to microtubule inhibitor docetaxel. These lead us to surmise that the function of CHFR is regulated by a different mechanism from that controlling the protein stability of CHFR. Recently, Plafker et al. proposed a new level of control to the ubiquitination[35]. E2 conjugating enzymes can also interact with importin-11, resulting in nuclear localization of E2 enzymes that facilitates ubiquitination of proteins by nuclear E3 ubiquitin ligases. The nuclear-cytoplasmic shuttling was observed in only one class, that is, class-III E2 enzymes which consist of UBE2E1, UBE2E2 and UBE2E3. It is noteworthy that UBE2E3 was included in the E2 enzymes isolated by two-hybrid screen using CHFR as bait. This raises the possibility that UBE2E3 importation into nucleus depends on cell cycle or mitotic stress, and thus causes ubiquitination of proteins by cooperation with nuclear CHFR. Further studies are warranted.

Our finding that gadd34 interacts with CHFR brought together two molecules that were previously thought unrelated. GADD34, like GADD45 and GADD153, was originally discovered as an UV-inducible transcript in Chinese hamster ovary cells [36]. Later studies demonstrated a correlation between the onset of apoptosis and GADD34 expression in selected cell lines following not only ionizing irradiation but also treatment with the alkylating agent[37], amino acid deprivation and several endoplasmic reticulum (ER) stresses [38, 39]. Our analysis indicated that the mRNA expression of gadd34 was almost unchanged within 24 hr after the docetaxel treatment. Even if gadd34 is involved in response to mitotic stress, the context of the molecular mechanism in which gadd34 functions is different from that in response to typical genotoxic stresses which induce mRNA expression of gadd34.

More recently, it has become apparent that gadd34 is also involved in recovering synthesis of endoplasmic reticulum (ER) client protein in stressed cells[32, 39-41]. Gadd34 shares carboxyl region similarity with a viral protein, HSV1 ICP 34.5, which is a virulence factor that blocks the premature shutoff of protein synthesis in HSV1-infected neuroblastoma cells [32]. Similar to the ICP34.5, gadd34 binds to the type 1 protein serine/threonine phosphatase (PP1), and activates the ability of PP1 to dephosphorylate eIF-2a, allowing most protein synthesis to resume [39, 40]. When the mammalian cells encounter stresses that impinge upon the normal folding of proteins in the ER, unfolded protein response (UPR) occurs to protect the cells, otherwise apoptotic pathways are activated to destroy the damaged cell[41]. UPR requires upregulation of ER chaperones and folding enzymes induced by PP1-mediated dephosphorylation of eIF-2 a. PP1 also regulates a variety of cellular processes through the dephosphorylation of dozens of substrates, including Aurora A[42]. Although gadd34 is unlike substrate for CHFR, it can not be ruled out that CHFR regulates (ubiquitinates) PP1 via gadd34 bridging and is involved in cell cycle regulation or mitotic response.

Antimicrotubule agents including docetaxel are potent promoters of apoptosis in cancer cells. Several groups have reported that microtubule inhibitors induce phosphorylation of Bcl-2 that inactivates anti-apoptotic capacities of Bcl-2 and are thus involved in apoptosis [43, 44]. MAP kinase JNK is responsible for the phosphorylation of mitochondrial Bcl-2 in mitotic arrested cells, and is involved in apoptosis induced by microtubule inhibitors [43]. On the other hand, Brichese et al. reported that PP1 forms a tripartite complex together with JNK and Bcl-2 at the mitchondrial level[45, 46]. PP1 dephospholylates

Bcl-2 in interphasic cells, however it dissociates from Bcl-2 after the mitotic stress induced by microtubule inhibitors[45]. Based on the notion that PP1 suppresses apoptosis by dephosphorylating Bcl-2, CHFR may positively regulate PP1 to dephosphorylate Bcl-2 via gadd34, and result in suppression of apoptosis. The reason for this is that cancer cells expressing CHFR is more resistant to microtubule inhibitors compared with those lacking expression of CHFR. From the view point of cell cycle, PP1 is also involved in Bcl-2 dephosphorylation after prolonged mitotic arrest induced by paclitaxel, and exit from mitosis due to mitotic slippage[45]. In this context, CHFR may negatively regulate PP 1 via gadd34, and thus cancer cells not expressing CHFR showed increased PP1 activity, resulting in mitotic slippage and possible mitotic catastrophe[47] induced by microtubule inhibitor.

In conclusion, our analysis indicated that gadd34 promoted non-proteolytic autoubiquitination of CHFR; the interaction with CHFR occurred in the presence of microtubule inhibitor and the colocalization of CHFR and gadd34 was linked to cell death. However, the meaning of autoubiquitination of CHFR induced by gadd34 remains unsolved. Focusing on the idea that CHFR preferentially promotes non-proteolytic ubiquitination, there might be other target proteins subjected to non-proteolytic ubiquitination by CHFR, besides CHFR itself. Detection of substrates or effectors that are directly regulated by CHFR is an important subject for future research, in order to further elucidate the molecular mechanisms underlying sensitivity of cancer cells to microtubule inhibitors.

ACKNOWLEDGMENT

This study was supported in part by Grants-in-Aid for Scientific Research on Priority Areas from the Ministry of Education, Culture, Sports, Science, and Technology (to H.M., M.T. and T.T.), the Akiyama foundation (to T. T.) and Mitsui Life Social Welfare Foundation (to T.T. and M.T.) .

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(Accepted for publication, Dec. 28, 2006)