The Auto-ubiquitylation of E3 Ubiquitin-protein Ligase Chfr at G2 Phase Is Required for Accumulation of Polo-like Kinase 1 and Mitotic Entry in Mammalian Cells*§

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The E3 ubiquitin-protein ligase Chfr is a mitotic stress checkpoint protein that delays mitotic entry in response to microtubule damage; however, the molecular mechanism by which Chfr accomplishes this remains elusive. Here, we show that Chfr levels are elevated in response to microtubule-damaging stress. Moreover, G2/M transition is associated with cell cycle-dependent turnover of Chfr accompanied by high auto ubiquitylation activity, suggesting that regulation of Chfr levels and auto-ubiquitylation activity are functionally significant. To test this, we generated Chfr mutants Chfr-K2A and Chfr-K5A in which putative lysine target sites of auto ubiquitylation were replaced with alanine. Chfr-K2A did not undergo cell cycle-dependent degradation, and its levels remained high during G2/M phase. The elevated levels of Chfr-K2A caused a significant reduction in phosphohistone H3 levels and cyclinB1/Cdk1 kinase activities, leading to mitotic entry delay. Notably, polo-like kinase 1 levels at G2 phase, but not at S phase, were ∼2–3-fold lower in cells expressing Chfr-K2A than in wild-type Chfr-expressing cells. Consistent with this, ubiquitylation of Plk1 at G2 phase was accelerated in Chfr-K2A-expressing cells. In contrast, Aurora A levels remained constant, indicating that Plk1 is a major target of Chfr in controlling the timing of mitotic entry. Indeed, overexpression of Plk1 in Chfr-K2A-expressing cells restored cyclin B1/Cdk1 kinase activity and promoted mitotic entry. Collectively, these data indicate that Chfr auto ubiquitylation is required to allow Plk1 to accumulate to levels necessary for activation of cyclin B1/Cdk1 kinase and mitotic entry. Our results provide the first evidence that Chfr auto ubiquitylation and degradation are important for the G2/M transition.

The E3 ubiquitin-protein ligase Chfr (checkpoint with fork-associated domain and RING finger) has been identified as a novel poly(ADP-ribose)-binding zinc finger (PBZ) motif-containing protein (19). Introducing mutations in the PBZ motif of Chfr or inhibition of poly(ADP-ribose) synthesis leads to abrogation in its antephase checkpoint function. A series of earlier studies has shown that the RING finger domain of Chfr, which confers E3 ligase activity, is required for its auto ubiquitylation and subsequent checkpoint function (10–13). In Xenopus laevis extracts, Chfr targets polo-like kinase (Plk)3 for proteasome-dependent degradation (14), which in turn stalls activation of cyclin B-associated Cdc2 kinase. However, other studies suggest that Chfr-mediated non-canonical signaling rather than proteasome-mediated destruction of target substrates is important in the response to mitotic stress (11, 12, 15). Moreover, Plk1 expression in human cell lines does not always correlate with reduced Chfr levels (16, 17), suggesting that alternative pathways to modulate the Chfr checkpoint function may exist in mammals. Accordingly, ubiquitylation-mediated signaling and activation of downstream p38 kinase but not proteasome-dependent degradation by Chfr is reported to be necessary for the antephase checkpoint (18) and exclusion of cyclin B from the nucleus by Chfr delays cell cycle progression in response to microtubule damage (17). Modification of Chfr activity by phosphorylation or ADP-ribosylation may also play a critical role in the checkpoint function of Chfr. Chfr undergoes phosphorylation by protein kinase B (PKB/Akt) upon DNA damage, and expression of a nonphosphorylatable mutant of CHFR results in reduction of levels of Plk1 and inhibition of mitotic entry (15). Chfr has been identified as a novel poly(ADP-ribose)-binding zinc finger (PBZ) motif-containing protein (19). Introducing mutations in the PBZ motif of Chfr or inhibition of poly(ADP-ribose) synthesis leads to abrogation in its antephase checkpoint function. The contradictory findings and whether and/or how the reported regulations of Chfr expression level and activity are interconnected remain to be resolved.

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3 The abbreviations used are: Plk, polo-like kinase; CIP, calf intestinal phosphatase; DTB, double thymidine block; PI, propidium iodide.
Role of Auto-ubiquitylation of Chfr

Here, we have demonstrated that modulation of the Chfr expression level is the key factor determining its checkpoint function. We have shown that Chfr levels are elevated when the checkpoint is activated upon microtubule stress. In addition, cell cycle-dependent ubiquitylation and degradation of Chfr at G2 phase is crucial for mitotic entry. By utilizing a Chfr-K2A mutant lacking putative auto-ubiquitylation target sites, we have demonstrated that accumulation of Chfr protein at G2 phase, but not in S phase, promotes degradation of Plk1, leading to delayed entry into mitosis. Thus, our findings provide the first demonstration that Chfr auto-ubiquitylation activity and degradation are important for the cell cycle and checkpoint functions of Chfr.

EXPERIMENTAL PROCEDURES

Plasmid and Antibodies—A full length of FLAG-tagged Chfr (p3xFLAG-Chfr) was used as the initial construct (13). To generate a FLAG-Chfr ΔRF mutant plasmid, Chfr cDNA lacking the 48 amino acids (EETLTCICQDLLHDCVSLQPCMH-/H11032-TATTAGGACAAGGCTGGTGGGCAC-3) was subcloned into p3xFLAG-CMV-7.1 (Sigma). A FLAG-Chfr TFCAACYSGWMERSSLCPTCRCPV) was subcloned into the 48 amino acids (EETLTCICQDLLHDCVSLQPCMH-/H11032-TATTAGGACAAGGCTGGTGGGCAC-3′; reverse, 5′/H11032-CGCAGTTGAGTGGGGACCTTGA-3′); the two PCR fragments were digested with XbaI and AfeI and inserted into FLAG-Chfr (WT), which was digested with AfeI and EcoNI. For FLAG-Chfr K2A, FLAG-Chfr K3A, and FLAG-Chfr K5A, PCR was performed using a primer set for FLAG-Chfr K2A (forward, 5′/H11032-AAATCTAGAACCAGTTGCTGCAGCATGTCTTGAGTG-3′; reverse, 5′/H11032-GGCCAGCATGTTGCTGCAGCTATGAGAGGTGGGGACAAATCTAGAACCAGTTGCTGCAGCATGTCTTGAGTG-3′). PCR fragments were digested with XbaI and AfeI and inserted between AvrII and AfeI of FLAG-Chfr (WT). PCR fragments were digested with XbaI and AfeI and inserted between AvrII and AfeI. PCR fragments were digested with XbaI and AfeI and inserted between AvrII and AfeI. PCR fragments were digested with XbaI and AfeI and inserted between AvrII and AfeI. PCR fragments were digested with XbaI and AfeI and inserted between AvrII and AfeI.

In Vivo and in Vitro Ubiquitylation Assay—HeLa cells were incubated in thymidine containing DMEM for 20 h and released from the thymidine block for 8 h. During the first thymidine-free incubation, cells were transfected with His-ubiquitin and FLAG-Chfr (wild-type or K2A mutant). After the DTB, cells were allowed to progress by G2/M phase. Cells were treated with 2 μM MG132 for 6 h before harvest. Whole cell extracts were prepared by E1A lysis buffer (50 mM HEPES (pH 7.0), 150 mM NaCl, 0.1% Nonidet P-40, 5 mM EDTA, 1 mM DTT, and protease inhibitors). Lysates were clarified by high speed centrifugation and quantified by using a Bradford reagent (Bio-Rad). The same protein lysates were immunoprecipitated with anti-FLAG antibody coupled to protein A-Sepharose beads for 1 h at 4°C. The immune complexes were washed four times with lysis buffer thoroughly. The samples were dissolved in 2× Laemmli sample buffer, boiled for 5 min, and separated by SDS-PAGE. The analysis of ubiquitylation was performed by immunoblotting using anti-ubiquitin antibody (Santa Cruz). For the in vitro ubiquitylation assay, the immunoprecipitates obtained by using anti-FLAG antibody were thoroughly washed four times and incubated with E1 (0.5 μg), UbcH5b (0.5 μg), and ubiquitin (5 μg) in buffer containing 20 mM HEPES (pH 7.3), 5 mM ATP, 10 mM MgCl2, 1 mM DTT, and 2.5 μM MG132 at 37 °C for 1 h. The analysis of ubiquitylation was performed by immunoblotting using anti-ubiquitin antibody. Alternatively, the immunoprecipitates were pretreated with 60 units of calf intestinal phosphatase (CIP) for 1 h at 37°C and subjected to ubiquitin conjugation in vitro.

Immunocytochemistry and Confocal Microscopy—Cells were seeded on coverslips and fixed with a mixture of acetone/methanol (1:1) solution for 10 min. The fixed cells were preincubated in blocking solution (1% bovine serum albumin in PBS) for 1 h followed by incubation with anti-FLAG antibody overnight at 4°C. The cells were then washed and probed with fluorescence-conjugated secondary antibody for 1 h. Finally, coverslips were submerged in PBS containing 4′,6-diamidino-2-phenylindole (DAPI, Molecular Probes) and mounted with Vectashield (Vector Laboratories) for microscopic observation. FLAG-tagged proteins were detected with Cy3-conjugated anti-rat IgG antibody (Molecular Probes). Images were captured under confocal microscopy (LSM510, Zeiss, Germany).

In Vivo Cell Cycle Progression—Chang cells were cultured in DMEM/F-12 (Sigma) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% antibiotic-antimycotic (Invitrogen) in CO2 incubator. Chang cells were cultured in DMEM (Invitrogen), and T24 and DLD1 cells were in RPMI 1640 with 10% FBS. Chang and HeLa Cells were synchronized at the G1/S boundary using the double thymidine block (DTB) method as previously described (20). HeLa cells were incubated with 2 mM thymidine (Sigma) for 20 h followed by thymidine-free incubation for 8 h and subjected to the second thymidine incubation for 14 h. Chang cells were treated with 2 mM thymidine for 16 h, released for 8 h, and then treated with second thymidine for 16 h. Cells were washed to remove thymidine with PBS and allowed to progress to G2/M phase. To obtain S phase-arrested cells, cells were allowed to grow in 2 mM hydroxyurea-containing media. For isolation of cells in G2 and mitotic phase, cells were treated with 100 ng/ml nocodazole for 12 h, and floating mitotic cells and residual adherent cells were separately collected. Alternatively, G2 cells were obtained by treatment with 100 μM roscovitine (Sigma).

Flow Cytometry and Mitotic Index Analyses—For analysis of cell cycle profiles, cells released from trypsinization, washed twice with PBS, and fixed in 70% ethanol on ice for 30 min. Fixed cells were washed twice with PBS and resuspended in solution containing propidium iodide (PI) and RNase A. PI-stained cells were analyzed for their DNA content by FACSCalibur (BD Biosciences), and cell cycle distribution percentage was calculated by Cell WinMDI Version 2.8 software. Mitotic index was determined by counting the cells with mitotic condensed chromatin, which were stained with aceto-orcein (Sigma) under light microscope. For each group, ~500 cells were analyzed.
In Vitro Kinase Assay and Immunoblotting—HeLa cells were synchronized at the G1/S boundary by the DTB method. During the DTB, cells were transfected with the FLAG-Chfr (WT) or FLAG-Chfr K2A. At each indicated time, cells were harvested and lysed in the lysis buffer (10 mM Tris-Cl (pH 7.4), 150 mM NaCl, 1% Nonidet P-40, 1% SDS, 0.5% deoxycholic acid and 1 mM EDTA) containing protease and phosphatase inhibitors (2 μg/ml aprotinin, 2 μg/ml leupeptin, 2 μg/ml pepstatin, and 50 μg/ml phenylmethylsulfonyl fluoride). The same protein lysates were immunoprecipitated with anti-cyclin B1 antibody coupled to protein G-Sepharose beads for 3 h at 4 °C. Beads were washed twice with lysis buffer and subsequently with kinase buffer (50 mM Tris-Cl (pH 7.5), 10 mM MgCl2, 1 mM DTT). Phosphorylation reaction was carried out in 20 μl of kinase buffer supplemented with 50 μg/ml histone H1 (Roche Diagnostics) and 10 Ci/mmole of [γ-32P]ATP at 37 °C for 30 min. The samples were dissolved in Laemmli sample buffer, boiled for 5 min, separated by SDA-PAGE, and analyzed by autoradiography. For immunoblotting, lysates were boiled, separated with SDS-PAGE, and transferred onto polyvinylidene difluoride membranes (Millipore). The immunoblots were visualized by enhanced chemiluminescence system (Amersham Biosciences).

Statistical Analyses—The error bars on the figures represent the mean ± S.D. of the independent determinations. The statistical analysis was performed with two-sided unpaired t tests used to assess statistical significance. Differences with p values of <0.05 were considered to be statistically significant.

RESULTS

Chfr Levels Are Elevated in Response to Mitotic Stress in Mammalian Cells—Because the checkpoint function of Chfr primarily relies on Chfr levels, we hypothesized that Chfr levels might be changed in response to microtubule stress (3). To address this, we transfected cells of the DLD1 colon carcinoma cell line, which do not express Chfr protein, with a vector-encoding FLAG-tagged wild-type Chfr (FLAG-Chfr or FLAG-Chfr WT). Twenty-four hours after transfection, we induced mitotic stress by exposing cells to nocodazole (100 ng/ml) and determined the level of Chfr by immunoblotting. Consistent with previous findings (3), a low percentage of Chfr-transfected cells was mitotic, as evidenced by low levels of phosphohistone H3, a well known mitotic marker, 16 h after nocodazole treatment (Fig. 1A). Notably, nocodazole treatment led to a ~2–3-fold increase in Chfr levels. To confirm whether this phenomenon can be generalized to other cells, we tested two other cell lines, T24 and HeLa, by transfecting FLAG-Chfr WT. In both cell types, Chfr protein levels were significantly increased after a 16-h exposure to nocodazole (Fig. 1B). This is the first indication that Chfr levels are elevated in response to mitotic stress.

These findings raised the possibility that the elevation in Chfr levels induced by microtubule stress contributes to the activation or maintenance of the checkpoint, serving as a regulatory factor for mitotic entry. If so, Chfr levels should differ between cells that remain in G2 phase and those that enter mitosis. To test this, we separately collected floating (mitotic) and adherent HeLa cells after treating with nocodazole for 12 h and analyzed Chfr expression. In adherent cells, the G2-specific marker CENP-F (centromere protein F) was localized to the nucleus, indicating that these cells are in G2 phase (21, 22). By contrast, CENP-F was localized to the kinetochore in floating cells, indicating that these cells are in the mitotic phase (supplemental Fig. 1A). Notably, Chfr levels were reduced in mitotic cells but remained high in G2 phase cells (Fig. 1C), supporting our hypothesis. Taken together, our results indicate that Chfr levels play a role in controlling mitotic entry under microtubule stress conditions.

Cell Cycle-dependent Degradation of Chfr Protein by Auto-ubiquitylation—Assuming that Chfr levels control mitotic entry, as suggested by our data, we hypothesized that the steady-state levels of Chfr would be regulated during the cell cycle, especially before mitotic entry. To test this, we synchronized Chfr-transfected HeLa cells at the G1/S boundary using the DTB method and then allowed synchronized cells to reenter the cell cycle by releasing them into fresh medium. A flow cytometry analysis revealed that ectopic expression of Chfr did not significantly affect cell-cycle progression profiles; two-thirds of cells were in G2/M phase at R10 (10 h after DTB release) and partly reentered G1 phase at R12 (supplemental Fig. 1B). As shown in Fig.
A, Chfr expression levels were high in G1/S phase (R0) cells but were markedly decreased during G2-M phase (R9–R12). We also monitored cell cycle-dependent changes in Chfr levels in cells synchronized at each phase by chemical reagents. S phase-arrested cells were obtained by treatment with hydroxyurea (HU, 2 mM) for 18 h and roscovitine (Ros, 100 μM) for 5 h at R7 (7 h after DTB release). The Chfr expression levels were determined by immunoblotting.

B, HeLa cells were synchronized at S or G2 phase by treatment with hydroxyurea (HU, 2 mM) for 18 h and roscovitine (Ros, 100 μM) for 5 h at R7 (7 h after DTB release). The Chfr expression levels were determined by immunoblotting.

C, HeLa cells were transfected with FLAG-Chfr (WT) followed by treatment of second thymidine for 14 h. Asynchronous (Asy) and synchronized cells at the indicated time points after the release from DTB were harvested and analyzed by immunoblotting.

D, HeLa cells co-transfected with FLAG-Chfr and His-ubiquitin (Ub) were synchronized at each phase by treatment with hydroxyurea and roscovitine as described above and followed by treatment with MG132 (2 μM) for 6 h before harvest. The ubiquitylation of Chfr was evaluated by anti-Ub antibody after lysates were immunoprecipitated (IP) with anti-FLAG antibody.

E, HeLa cells were co-transfected with FLAG-Chfr and His-ubiquitin (Ub) were synchronized at each phase by treatment with hydroxyurea and roscovitine as described above and followed by treatment with MG132 (2 μM) for 6 h before harvest. The ubiquitylation patterns were determined by immunoblotting.

F, the immunoprecipitates obtained with anti-FLAG antibody were incubated with 60 units of CIP for 1 h and subjected to in vitro ubiquitylation as described in E.

FIGURE 2. Cell cycle-dependent degradation of Chfr protein by auto-ubiquitylation. A, HeLa cells were synchronized at the G1/S boundary by the DTB method. After first thymidine treatment, cells were transfected with the FLAG-Chfr (WT) followed by treatment of second thymidine for 14 h. Asynchronous (Asy) and synchronized cells at the indicated time points after the release from DTB were harvested and analyzed by immunoblotting. B, HeLa cells were synchronized at S or G2 phase by treatment with hydroxyurea (HU, 2 mM) for 18 h and roscovitine (Ros, 100 μM) for 5 h at R7 (7 h after DTB release). The Chfr expression levels were determined by immunoblotting.
We next examined whether the reduction in Chfr levels at G2 phase is mediated by variations in ubiquitylation activity during the cell cycle. Accordingly, we co-transfected HeLa cells with FLAG-Chfr WT and an expression plasmid for His-tagged ubiquitin and collected cells from each phase of the cell cycle as noted in Fig. 2B. Cell lysates were immunoprecipitated using an anti-FLAG antibody followed by Western blotting with an anti-ubiquitin antibody. Interestingly, extensively ubiquitylated Chfr was observed exclusively in G2-arrested cells and not in cells at S phase and asynchronously growing cells (Fig. 2D), indicating that Chfr proteins are degraded at G2 phase in an ubiquitylation-dependent manner. To further verify our findings, we carried out an in vitro ubiquitin conjugation assay (11, 25). HeLa cells were transfected with FLAG-Chfr and collected cells from each phase of the cell cycle. The immunoprecipitates obtained by anti-FLAG antibody were incubated with E1, UbcH5b as E2, ubiquitin, and ATP, and ubiquitylation patterns were analyzed by immunoblotting. We found that in vitro auto-ubiquitination activities of Chfr in cells of G2 and mitotic phase were higher than that of G1/S (Fig. 2, E and F). In addition, pre-treatment with phosphatase reduced the increased auto-ubiquitination activities of Chfr in G2 and mitotic phase (Fig. 2F), indicating that cell cycle-dependent changes of Chfr auto-ubiquitination is likely controlled by phosphorylation. Taken together, these results demonstrate that Chfr exhibits a cell cycle-dependent turnover that is regulated by its auto-ubiquitination activity.

**Substitution of Alanine for Putative Auto-ubiquitylation Target Lysine Residues**—Next, we asked whether the cell cycle-dependent turnover of Chfr protein is necessary for cell-cycle progression. Because the loss of Chfr ubiquitin ligase activity in FLAG-Chfr ΔRF or FLAG-ChfrL306A might affect cell-cycle progression due to loss of stability and failure to degrade extrinsic target substrates, we attempted to produce a mutant Chfr construct that did not contain target sites for auto-ubiquitylation but still retained its ligase activity. Previous studies proposed that a Chfr fragment comprising amino acids 281–375, which contains the entire RING finger E3 ligase domain, may target sites in flanking regions for polyubiquitination (26). We, therefore, generated three mutant constructs in which putative auto-ubiquitylation target lysine residues were replaced with alanine: FLAG-Chfr K2A, containing K384A and K393A substitutions; FLAG-Chfr K3A, containing three Lys-to-Ala substitutions (K257A/K258A/K259A); and FLAG-Chfr K5A, containing all five substitutions (Fig. 3A). After transfecting HeLa cells with the same amount of plasmid DNA encoding either of these alanine-substitution mutants or FLAG-Chfr WT, we found that the basal expression level of each alanine-substitution mutant, determined by Western blotting, was higher than that of wild-type Chfr (Fig. 3B), suggesting that these mutants are likely resistant to ubiquitylation. An examination of subcellular localization by immunofluorescence staining showed that both FLAG-Chfr K2A and FLAG-Chfr WT were present in the nucleus (Fig. 3C). In contrast, both FLAG-Chfr K3A and FLAG-Chfr K5A were mainly detected in the cytoplasm. Because Chfr was originally defined as a nuclear protein (8) that appears to be derived from nuclear localization signal at amino acid residues 257–259 (27), the FLAG-Chfr K2A mutant was chosen for further study because it retained a wild-type-like subcellular localization.

**Stabilization of Chfr in Vivo by Mutation of Putative Auto-ubiquitylation Target Sites**—We first examined whether the putative auto-ubiquitylation target mutant FLAG-Chfr K2A showed cell cycle-dependent degradation. A significant reduction in FLAG-Chfr WT protein was consistently observed during G2/M transition (Fig. 4A). In contrast, the protein level of FLAG-Chfr K2A did not change from R0 to R12, suggesting that FLAG-Chfr K2A escaped from auto-ubiquitylation and subsequent degradation. Interestingly, histone H3 phosphorylation was markedly weaker in cells expressing FLAG-Chfr K2A, indicating that mitotic entry was hindered in these cells. Next, we compared the ubiquitylation status of FLAG-Chfr WT and the FLAG-Chfr K2A mutant in vivo after co-transfection of His-tagged ubiquitin and Chfr expression vectors (Fig. 4B). We observed a significant increase in ubiquitylation on FLAG-Chfr WT during G2/M progression (R8 and R10), whereas little ubiquitylation was observed at G1/S (R0). In contrast, ubiquitylation on FLAG-Chfr K2A was greatly suppressed at R8 and R10 (Fig. 4B), indicating that Lys-384 and Lys-393 are indeed important ubiquitylation target sites. Collectively, our data demonstrate that the auto-ubiquitylation activity of Chfr is important for controlling Chfr levels during G2/M transition. Thus, mutation of putative auto-ubiquitylation target sites of Chfr abrogates cell cycle-dependent Chfr degradation, leading to high Chfr levels during the cell cycle.

**Delay of Mitotic Entry by Abrogation of Cell Cycle-dependent Degradation of Chfr**—It was notable that G2/M transition appeared to be hindered in cells maintaining high Chfr levels (Fig. 4A). To further investigate this, we transfected HeLa cells with two different amounts of FLAG-Chfr expression plasmid...
and examined subsequent effects on G2/M transition. We found that a higher concentration (4 μg) of FLAG-Chfr WT reduced phosphohistone H3 levels (Fig. 5A), an effect that was almost completely abolished by co-transfection with 4 μg of FLAG-Chfr K2A, strongly indicating that Chfr levels are critical for controlling G2/M transition. Next, we examined cell-cycle profiles during G2/M progression in these cells after synchronization using the DTB method. Flow cytometry analyses revealed that G2/M phase cell populations at R10 were similar among pCDNA-transfected control cells and FLAG-Chfr WT- and FLAG-Chfr K2A-transfected cells (Fig. 5B). However, at R12, ~90% of control cells reentered the G1 phase, whereas 17 and 30% of FLAG-Chfr WT- and FLAG-Chfr K2A-transfected cells, respectively, remained in G2/M phase. In fact, an analysis of the mitotic index, determined by staining cells with aceto-orcein, showed that the percentage of mitotic cells at R10 was significantly lower in FLAG-Chfr K2A-expressing cells than in FLAG-Chfr WT- or vector (control)-expressing cells (Fig. 5C). Control- and FLAG-Chfr WT-expressing cells reached a peak of mitosis 10 h after DTB release, at which time 25–30% of cells were in mitosis; however, the mitotic index of FLAG-Chfr K2A-expressing cells was as low as 15%. Moreover, a higher concentration (4 μg) of Chfr-K2A further decreased the mitotic index to less than 10%, demonstrating a clear inverse relationship between Chfr levels and mitotic index. To understand the underlying molecular mechanism, we determined the level of cyclin B1-associated kinase activity, which is critical for G2/M transition. As shown in Fig. 5D, cyclin B1-associated kinase activity in cells expressing FLAG-Chfr WT was high at R8 and was further increased at R10. In contrast, cyclin B1-associated kinase activity in FLAG-Chfr K2A-expressing cells remained low from R8 to R10 and became elevated at 10.5 h (Fig. 5D). Thus, our data clearly demonstrate that high expression levels of Chfr at G2 phase suppress cyclin B1-associated kinase activity, thereby causing a delay in mitotic entry. This is the first indication that balanced Chfr level at G2 phase is a critical factor in controlling the timing of mitotic entry.

**Plk1 Acts as a Downstream Mediator of Chfr in Controlling the Timing of Mitotic Entry in Mammalian Cells**—Although it has been previously reported that Chfr mediates the degradation of Plk1 in *Xenopus* cell-free extracts (13), these findings have remained controversial in mammalian systems (16). Plk1 is an important cell-cycle regulator that determines the timing of mitotic entry by controlling cyclin B1-associated kinase activity (28–30). Aurora A kinase, another important target substrate of Chfr, also modulates cyclin B1-associated kinase activities, mainly at centrosomes (31, 32). We, therefore, investigated whether the levels of Plk1 and Aurora A showed any correlations with Chfr levels during the cell cycle. HeLa cells were transfected with FLAG-Chfr or mutant forms of Chfr (K2A, K5A, RF) and synchronized at S phase and G2 phase by hydroxyurea and roscovitine, respectively. The expression levels of Plk1 and Aurora A were higher in G2-arrested cells than in S phase-arrested cells (Fig. 6A). Notably, at G2 phase, Chfr expression levels in cells expressing either FLAG-Chfr K2A or FLAG-Chfr K5A were 2–3-fold higher than in FLAG-Chfr
WT-expressing cells (Fig. 6A), and Plk1 levels were reduced by half (Fig. 6A, right panel). In contrast, the Plk1 level in cells expressing FLAG-Chfr ΔRF, which lacks Chfr ubiquitin ligase activity, was similar to that in FLAG-Chfr WT-expressing cells, indicating that Chfr-dependent Plk1 degradation does not occur. Thus, the data showed an inverse relationship between Chfr and Plk1 levels at G2 phase but not in S phase. In contrast, we did not observe any significant changes in Aurora A levels, indicating that Chfr-mediated Aurora A degradation does not occur in a cell cycle-dependent manner.

Next, we examined whether Plk1 was a genuine G2 phase-specific substrate of Chfr by carrying out an in vivo ubiquitylation assay. HeLa cells were co-transfected with a His-tagged ubiquitin expression construct and different Chfr constructs, after which whole-cell lysates were immunoprecipitated with anti-Plk1 antibody and then analyzed by Western blotting using an anti-ubiquitin antibody. As shown in Fig. 6B, in contrast, there was little, if any, Plk1 ubiquitylation in S phase-arrested cells. Notably, ubiquitylation on Plk1 was strongly promoted at G2 phase in cells expressing FLAG-Chfr K2A, but neither FLAG-Chfr WT nor FLAG-Chfr ΔRF (RING-dead) promoted Chfr-dependent ubiquitylation on Plk1 (Fig. 6B). The lower panel of Fig. 6B shows that equal amounts of Chfr and Plk1 (Input) were used for the assay.

Thus far, our data strongly suggest that FLAG-Chfr K2A can induce a delay in G2/M transition by reducing Plk1 levels at G2 phase. Indeed, when Myc-tagged Plk1 was overexpressed in FLAG-Chfr K2A-expressing cells, we found a notable recovery of mitotic index at R10 (Fig. 6C). Consistent with this, cyclin B1-associated kinase activity was elevated by more than 2-fold at time points 10–10.5 h after DTB (Fig. 6D). Taken together, our data demonstrate that Plk1 is an important downstream target of Chfr ubiquitin ligase at G2 phase, and its level is critical for the activation of cyclin B1-associated kinase and mitotic entry.

DISCUSSION

In the present study we demonstrated that Chfr levels changed in a logical way to cope with different cellular microenvironments; they are elevated upon microtubule damage, serving as a cell-cycle checkpoint, and they are markedly reduced at G2 phase, relieving a break against entry into mitosis. Thus, the data presented here highlight the importance of Chfr levels in both checkpoint function and control of cell-cycle progression.
The auto-ubiquitylation activity of Chfr mediates cell cycle-dependent changes in Chfr levels. We demonstrated that reduced Chfr levels at G2 phase were accompanied by an increase in ubiquitylation on Chfr that was dependent on Chfr E3 ligase activity (Fig. 2). These findings partially reiterate those of a previous report (12) in that fluctuations in Chfr levels over the cell cycle were lost in cells expressing the FLAG-Chfr/H9004RF mutant, which lacks E3 ligase activity. Importantly, the auto-ubiquitylation-defective FLAG-Chfr K2A mutant did not show cell cycle-dependent changes. In fact, it effectively inhibited entry into mitosis (Fig. 5), suggesting that auto-ubiquitylation and degradation of Chfr at G2 phase is a prerequisite for proper cell-cycle progression into mitosis. Thus, Chfr not only acts as a mitotic stress checkpoint in the presence of microtubule damage but also controls cell-cycle progression at the G2/M transition. The fact that Chfr degrades at G2 phase helps to explain why ectopic expression of Chfr in cells that lack it does not usually cause cell-cycle delay. However, the robust elevation in Chfr levels in response to microtubule damage (Fig. 1), noted previously (18), confers a stronger gatekeeper function that prevents cells from entering mitosis. Notably, FLAG-Chfr/H9004RF-expressing cells entered mitosis without any delay, as determined by phosphohistone H3 levels (Fig. 2D), suggesting that ubiquitylation of target substrates is crucial in the control of cell-cycle progression. In addition, we addressed whether cell cycle-dependent Chfr degradation occurs in cells stably expressing Chfr. We established different clones that stably express wild-type Chfr or Chfr K2A mutant (FLAG-Chfr K2A-S). The FLAG-Chfr WT-S or FLAG-Chfr-S7 cells expressing wild-type Chfr showed cell cycle-dependent degradation,
whereas the FLAG-Chfr-K2A-S cells did not (supplemental Fig. 2, A and B). However, we noticed that cell cycle-dependent degradation of Chfr in stable cells was weakened probably due to low Chfr protein levels. In fact, when we compared the Chfr protein levels in three stable cell lines (FLAG-Chfr-S1, FLAG-Chfr-S7, FLAG-Chfr-S9) to those in transiently transfected cells, all of the stable cell lines showed much lower Chfr protein levels (upper panel of supplemental Fig. 2B). These consistent observations suggest that cells cannot tolerate high Chfr protein, and therefore, the cells proliferate/survive through controlling its auto-ubiquitination and/or hypermethylation on its own promoter (7–9).

There have been conflicting reports on whether Plk-1 is an important substrate of Chfr in Chfr-mediated cell-cycle delay. The first evidence for such a role came from the Xenopus system, where Chfr-dependent ubiquitylation of Plk1 was demonstrated. The authors showed that degradation of Plk1 by Chfr is responsible for the delay in mitotic entry because of inhibition of cyclinB/Cdc2 (13). However, overexpression of Chfr in human cells has not been shown to cause a reduction in Plk1 levels (16–18). In the present study overexpression of FLAG-Chfr WT tended to decrease Plk1 levels (Fig. 6A), but these differences were not significant. However, introduction of auto-ubiquitylation-defective mutants FLAG-Chfr K2A or FLAG-Chfr K5A into cells resulted in a significant reduction in Plk1 levels compared with those in cells expressing FLAG-Chfr WT. More intriguingly, these differences were only observed in G2 phase and not in S phase, indicating that Chfr-mediated Plk1 degradation occurs in a cell cycle-dependent manner. Thus, previous reports based on asynchronous cell cultures would be unable to detect an inverse relationship between Chfr and Plk1; our data, based on precisely synchronized cells, fully support the previous findings of the Xenopus system in which a well-characterized synchronization method was applied (13). In addition, Plk1 levels were similar in cells expressing FLAG-Chfr ΔRF, which lacks ubiquitin ligase activity, and FLAG-Chfr WT, both of which enter mitosis, supporting the interpretation that the Plk1 level is crucial in determining mitotic entry.

One key reasoning question that will need to be addressed by future research is what triggers activation of Chfr ubiquitin ligase at the G2 phase. It is reasonable to speculate that Chfr is post-translationally modified to achieve full E3 ligase activity. The possibility that Chfr is phosphorylated was initially suggested based on differences in the electrophoretic mobility of Chfr bands (11); Chfr phosphorylation has since been demonstrated in vivo and in vitro (15). In fact, we found that pretreatment with phosphatase reduced the increased auto-ubiquitylation activities of Chfr in G2 and mitotic phase (Fig. 2F), indicating that phosphorylation of Chfr controls its auto-ubiquitylation activity. It is also very unlikely that cyclin B/Cdk1 phosphorylates or modulates Chfr because FLAG-Chfr K2A is fully active in the presence of roscovitine, a cyclin B/Cdk1 inhibitor (Fig. 6, A and B). Notably, however, injection of active cyclin A/Cdk2 into PtK1 cells actually overrides the anaphase checkpoint in response to colcemide treatment (18), suggesting that cyclin A/Cdk2 may modulate Chfr activity through phosphorylation. In summary, our results provide the first evidence that Chfr auto-ubiquitylation and degradation is biologically significant in the cell cycle and checkpoint function of Chfr.

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