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The checkpoint protein Chfr is a ligase that ubiquitinates Plk1 and inhibits Cdc2 at the G2 to M transition

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The checkpoint protein Chfr delays entry into mitosis, in the presence of mitotic stress (Scolnick, D.M., and T.D. Halazonetis. 2000. *Nature*. 406:430–435). We show here that Chfr is a ubiquitin ligase, both in vitro and in vivo. When transfected into HEK293T cells, Myc–Chfr promotes the formation of high molecular weight ubiquitin conjugates. The ring finger domain in Chfr is required for the ligase activity; this domain auto-ubiquitinates, and mutations of conserved residues in this domain abolish the ligase activity. Using *Xenopus* cell-free extracts, we demonstrated that Chfr delays the entry into mitosis by negatively regulating the activation of the Cdc2 kinase at the G2–M transition. Specifically, the Chfr pathway prolongs the phosphorylated state of tyrosine 15 in Cdc2. The Chfr-mediated cell cycle delay requires ubiquitin-dependent protein degradation, because inactivating mutations in Chfr, interference with poly-ubiquitination, and inhibition of proteasomes all abolish this delay in mitotic entry. The direct target of the Chfr pathway is Polo-like kinase 1 (Plk1). Ubiquitination of Plk1 by Chfr delays the activation of the Cdc25C phosphatase and the inactivation of the Wee1 kinase, leading to a delay in Cdc2 activation. Thus, the Chfr pathway represents a novel checkpoint pathway that regulates the entry into mitosis by ubiquitin-dependent proteolysis.

Introduction

Cell cycle progression is monitored by checkpoint mechanisms to ensure the integrity of the genome and the fidelity of sister chromatid separation (Elledge, 1996). A cell cycle checkpoint that acts at prophase has been recently discovered (Scolnick and Halazonetis, 2000). In normal human cell lines, chromosome condensation is delayed in response to drugs that disrupt microtubule structure, such as taxol and nocodazole, suggesting a checkpoint that functions at the G2 to M transition (Jha et al., 1994). Although the exact defects sensed by this checkpoint mechanism have not been characterized, centrosome separation, which is inhibited in these cells, is a likely target of this checkpoint (Scolnick and Halazonetis, 2000). A key component of the checkpoint is the Chfr protein that delays chromosome condensation and nuclear envelope breakdown in response to mitotic stress induced by taxol or nocodazole. In four out of eight human tumor cell lines examined, the Chfr gene is either mutated or not expressed, and the Chfr checkpoint does not function. Ectopic expression of Chfr in these cells restores the cell cycle delay, indicating that Chfr is required for the checkpoint control (Scolnick and Halazonetis, 2000). The Chfr protein contains three separate domains, an NH_2-terminal forkhead-associated (FHA)* domain, a central ring finger (RF) domain, and a COOH-terminal cysteine-rich (CR) domain. Based on mutagenesis analysis, both the FHA and CR domains are required for the checkpoint function (Scolnick and Halazonetis, 2000). The importance of the RF domain in Chfr function is yet to be determined.

Progression from G2 to M is controlled by activation of the mitosis-promoting factor (MPF), a complex of the catalytic kinase subunit, Cdc2, and its positive regulatory subunit, cyclin B (King et al., 1994). As cells enter prophase, accumulation of mitotic cyclin B leads to formation of the Cdc2–cyclin B complex, which is then transported into the nucleus (Murray and Kirschner, 1989; Solomon et al., 1990). However, MPF is held in an inactive state by the

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*Abbreviations used in this paper: APC, anaphase-promoting complex/cyclosome; CR, cysteine-rich; FHA, forkhead-associated; GST, glutathione-*S*-transferase; LLnL, *N*-acetyl-Leu-Leu-norleucinal peptide; MPF, mitosis-promoting factor; Plk1, Polo-like kinase 1; RF, ring finger; SCF, Skp1/cullin/F-box complex; Ub, ubiquitin.

Wee1 and Myt1 kinases, which phosphorylate Cdc2 on tyrosine 15 and threonine 14 and inhibit MPF activity (Russell and Nurse, 1987; Draetta et al., 1988; Featherstone and Russell, 1991; Kornbluth et al., 1994; Mueller et al., 1995). Activation of the Cdc2 kinase and entry into prometaphase do not occur until the dual-specific phosphatase Cdc25C dephosphorylates Cdc2 (Russell and Nurse, 1986; Gautier et al., 1991; Kumagai and Dunphy, 1991, 1992; Osmani et al., 1991). Once activated, MPF triggers chromosome condensation, breakdown of the nuclear envelope, and formation of the mitotic spindle.

The activity of MPF is not only controlled by posttranslational modifications, such as phosphorylation and de-phosphorylation, but also by a ubiquitin-dependent proteolysis pathway (Fang et al., 1999). Cyclin B is degraded by such a pathway at late anaphase, which inactivates the Cdc2 kinase and allows cells to exit from mitosis.

Ubiquitin-dependent proteolysis involves cascade reactions of several enzymes, including a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a ubiquitin ligase (E3). In this series of enzymatic reactions, the critical enzyme is the ubiquitin ligase that links E2 to substrates and provides specificity for the ubiquitination pathway (Hershko and Ciechanover, 1998). A group of ubiquitin ligases contains the ring finger domain (Lorick et al., 1999; Huang et al., 2000; Joazeiro and Weissman, 2000). The prototype of this group of E3s is the proto-oncogene c-Cbl that targets receptor tyrosine kinases for ubiquitination and degradation (Joazeiro et al., 1999). The RF domain in c-Cbl directly interacts with E2, and c-Cbl functions to bring the E2–ubiquitin (Ub) conjugate adjacent to substrates (Yokouchi et al., 1999; Zheng et al., 2000). In the absence of substrates, the RF domain by itself has an auto-ubiquitination activity, a hallmark for this group of ubiquitin ligases (Joazeiro et al., 1999; Yokouchi et al., 1999).

There are at least two ubiquitin ligases involved in cell cycle control (King et al., 1996). The Skp1/cullin/F-box (SCF) ligase recognizes an inhibitor of cyclin-dependent kinase, sic1, and controls the G1–S transition (Krek, 1998; Willems et al., 1999). A second ubiquitin ligase, the anaphase-promoting complex/cyclosome (APC), controls both the separation of sister chromatids and the exit from mitosis by ubiquitinating multiple substrates, such as anaphase inhibitors and mitotic cyclin B (Fang et al., 1999). SCF and APC each contains a ring finger subunit (Roc1 and APC11, respectively). It has been shown that both Roc1 and APC11 auto-ubiquitinate and therefore have intrinsic ubiquitin ligase activity, although the recognition of substrates requires additional subunits (Ohta et al., 1999; Seol et al., 1999; Gmachl et al., 2000; Leverson et al., 2000). Furthermore, the RF domain in each protein is critical for ligase activity, because point mutations in RF domains abolish the ligase activity.

The checkpoint protein Chfr also contains a ring finger domain (Scolnick and Halazonetis, 2000), raising the possibility that Chfr may function as a ubiquitin ligase. We show here that, like Roc1 and APC11, Chfr auto-ubiquitinates and has intrinsic ubiquitin ligase activity. The RF domain is both necessary and sufficient for the auto-ubiquitination activity in vitro. Chfr also acts as a ubiquitin ligase in vivo, and Myc–Chfr immunopurified from HEK293T cells promotes the formation of high molecular weight ubiquitin conjugates. We characterized the specificity of Chfr toward ubiquitin-conjugating enzymes, and found that both Ubc4 and Ubc5, but not UbcH7 and UbcH10, function with the Chfr ligase. Furthermore, we developed a cell-free system to analyze the biological function of Chfr ligase. When added to *Xenopus* extracts, recombinant Chfr delays the activation of the Cdc2 kinase during the G2 to M transition; this delay is caused by a prolonged inhibitory phosphorylation of tyrosine 15 on Cdc2. The ubiquitin-dependent degradation by active Chfr ligase is required for the mitotic delay. The target of the Chfr ligase is the Polo-like kinase 1 (Plk1), and ubiquitination and degradation of Plk1 delays mitotic entry. Thus, Chfr represents a novel ubiquitin ligase involved in cell cycle regulation, and our biochemical analysis of the Chfr function in *Xenopus* extracts provides a molecular mechanism for Chfr-mediated checkpoint control at the G2 to M transition.

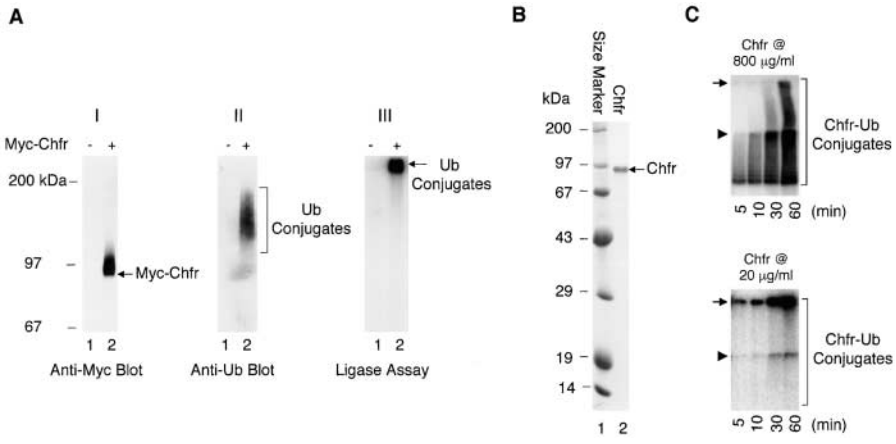

Figure 1. **Chfr is a ubiquitin ligase.** (A) Myc–Chfr (lanes 2) and control vector (lanes 1) were transfected into HEK293T cells and immunoprecipitated by an anti-Myc antibody. The immunoprecipitates were analyzed by Western blotting with an anti-Myc antibody (panel I) or with an anti-ubiquitin antibody (panel II). In addition, immunoprecipitates were incubated with radioactive ubiquitin in the presence of recombinant E1 and Ubc4 and assayed for ubiquitin ligase activity (panel III). The molecular weight markers for panels I–III are labeled on the left side of panel I. (B) Purified recombinant Chfr protein assayed by 12% SDS-PAGE. (C) Recombinant Chfr, at indicated final concentrations, was incubated with radioactive ubiquitin in the presence of E1 and Ubc4. The kinetics of the formation of the Chfr–Ub conjugates was assayed by 12% reducing SDS-PAGE. The arrows point to the wells of the stacking gel and the arrowheads indicate the junction between stacking and separation gels.

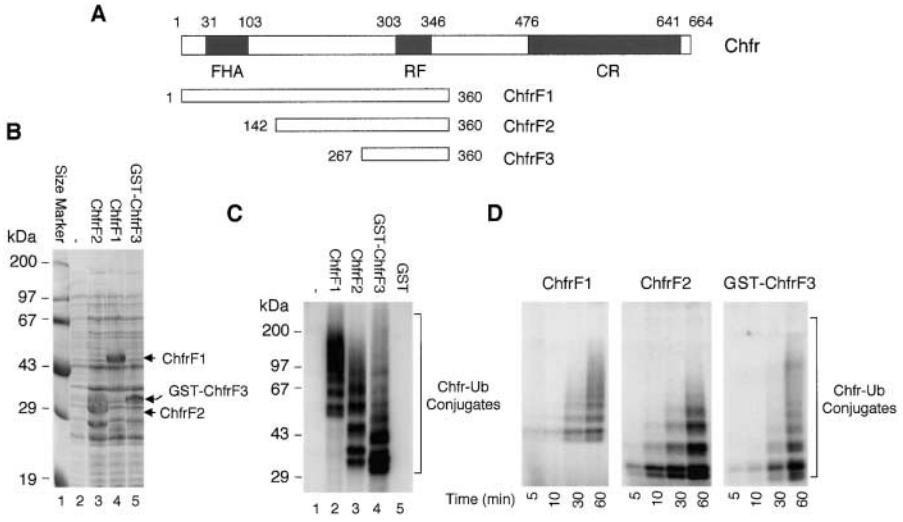

Figure 2. **The ring finger domain is sufficient to mediate the auto-ubiquitination reaction.** (A) A schematic diagram for the domain structure of the Chfr protein and the deletion constructs used in this paper. (B) Chfr deletion mutants were expressed in *E. coli* and their expression levels were assayed by 12% SDS-PAGE. Lane 2, lysates of *E.coli* without Chfr mutant proteins. (C) Equal amounts of *E. coli* lysates expressing various recombinant proteins were incubated with radioactive ubiquitin in the presence of recombinant E1 and Ubc4 and the formation of ubiquitin conjugates was analyzed by reducing SDS-PAGE. (D) Purified ChfrF1 (400 μg/ml final concentration), ChfrF2 (400 μg/ml), and GST–ChfrF3 (200 μg/ml) were incubated with radioactive ubiquitin in the presence of recombinant E1 and Ubc4 and the kinetics of ubiquitin conjugate formation was analyzed.

Results

Chfr is a ubiquitin ligase

To test whether Chfr is a ubiquitin ligase, we transfected a Myc-tagged *Chfr* gene into HEK293T cells (Fig. 1 A, panel I). Myc–Chfr was immunopurified by an anti-Myc antibody and assayed by Western blotting with an anti-ubiquitin antibody. The Chfr immunoprecipitate, but not the immunoprecipitate from control transfected cells, contained a ubiquitinated protein(s) (Fig. 1 A, panel II). It remains to be determined whether the ubiquitinated protein is Chfr itself or another protein associated with Chfr. Although the majority of the Myc–Chfr protein did not comigrate with the ubiquitinated species (Fig. 1 A, compare panels I and II), we cannot exclude the possibility that only a minor portion of Myc–Chfr was ubiquitinated, and this minor species escaped detection in panel I.

We next tested whether the Myc–Chfr immunoprecipitate had a ubiquitin ligase activity. The Myc–Chfr immunoprecipitate was incubated with radioactive ubiquitin in the presence of a recombinant ubiquitin activating (E1) and conjugating (Ubc4) enzyme (Fig. 1 A, panel III). The Chfr immunoprecipitate efficiently promoted the formation of high molecular weight ubiquitin conjugates that did not enter the stacking gel. Thus, the Chfr immunoprecipitate had ubiquitin ligase activity. Because it is possible that the Chfr immunoprecipitate may have contained additional proteins, we do not know from this experiment whether the ubiquitin ligase activity was directly derived from the Chfr protein, nor do we know the nature of the ubiquitinated products.

It has been shown recently that several ring finger–containing ubiquitin ligases can auto-ubiquitinate (Joazeiro et al., 1999; Joazeiro and Weissman, 2000). To determine whether the Chfr protein has intrinsic ubiquitin ligase activity, we examined the ability of recombinant Chfr to auto-ubiquitinate. The Chfr protein was expressed in Sf9 cells and purified to homogeneity (Fig. 1 B). When the Chfr protein was incubated with radioactive ubiquitin in the presence of the recombinant E1 and Ubc4, ubiquitin conjugates were efficiently formed (Fig. 1 C). These conjugates were resistant to reduction by DTT, suggesting that ubiquitin is conjugated through an isopeptide bond rather than a thioester bond. The formation of these ubiquitin conjugates was dependent on E1, Ubc4, and Chfr; omitting any one of these proteins resulted in no DTT-resistant conjugates (unpublished data). At a high Chfr concentration (top), we detected a series of ubiquitinated protein ladders, the fastest migrating species having a mobility very close to that of the recombinant Chfr, but different from that of E1 and Ubc4 (unpublished data). We conclude that these radioactive bands represent covalent conjugates between Chfr and labeled ubiquitin, and that Chfr acts as a ubiquitin ligase to auto-ubiquitinate in the presence of E1 and Ubc4. Similarly, we detected formation of Chfr–Ub conjugates with the Chfr protein expressed in *Escherichia coli* (unpublished data), thereby excluding the possibility that a contaminating protein from Sf9 cells may function as a ubiquitin ligase for Chfr.

At a high Chfr concentration (800 μg/ml; Fig. 1 C, top), we detected a ladder of ubiquitinated Chfr. Interestingly, at a lower Chfr concentration (20 μg/ml; Fig. 1 C, bottom), the majority of ubiquitin conjugates formed had such high molecular weights that they failed to enter the stacking gel. Only a minor portion of the conjugates was trapped between the stacking gel and the separating gel. No mono- or oligo-ubiquitin conjugates were detected. The different modes of Chfr reactivity were likely due to the relative ratio of Chfr to Ubc4 in these reactions, because the concentration of Ubc4 used in both panels was 10 μg/ml, a concentration twofold higher than the molar concentration of Chfr used in the bottom panel, but 20-fold lower than that of Chfr in the top panel.

The ring finger domain in Chfr is required for its ligase activity

We next examined whether the ring finger domain is required for the ubiquitin ligase activity of the Chfr protein. The domain structure of Chfr is illustrated in Fig. 2 A (Scolnick and Halazonetis, 2000). We constructed three deletion mutants, ChfrF1–3, which consist of various regions

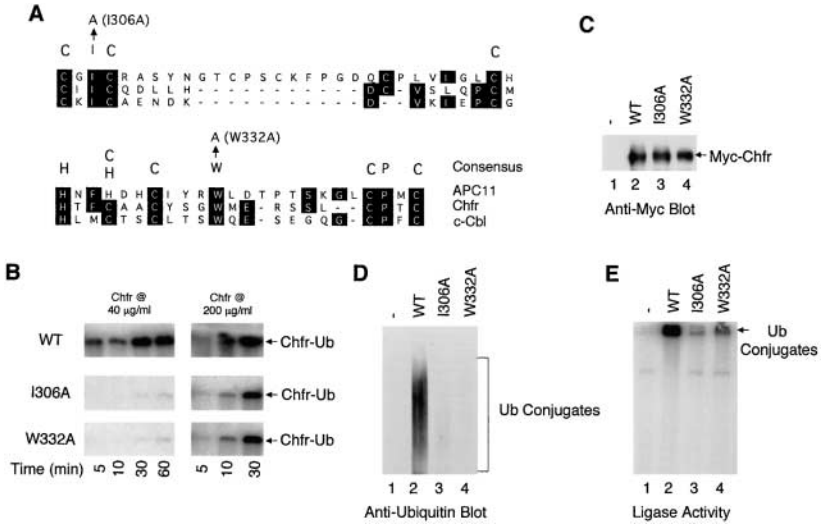

Figure 3. **The ring finger domain is required for the Chfr ligase activity.** (A) Alignment of the ring finger domains from APC11, Chfr, and c-Cbl. (B) Purified recombinant Chfr, ChfrI306A, and ChfrW332A, at concentrations indicated, were incubated with radioactive ubiquitin in the presence of E1 and Ubc4, and the kinetics of ubiquitin conjugate formation was followed by reducing SDS-PAGE. At the Chfr concentrations used here, all the ubiquitin conjugates stayed in the wells of the stacking gel and therefore only that portion of the gel is shown. (C–E) Myc–Chfr (lanes 2), Myc–ChfrI306A (lanes 3), Myc–ChfrW332A (lanes 4), and control vector (lanes 1) were transfected into HEK293T cells and immunoprecipitated by an anti-Myc antibody. The immunoprecipitates were analyzed by Western blotting with an anti-Myc antibody (C) or with an anti-ubiquitin antibody (D). In addition, immunoprecipitates were incubated with radioactive ubiquitin in the presence of recombinant E1 and Ubc4 and assayed for ubiquitin ligase activity (E). Arrow in E points to the wells of the stacking gel.

of the protein. All three fragments were expressed as soluble proteins in *E. coli* (Fig. 2 B). When the lysates of *E. coli* expressing mutant proteins were incubated with radioactive ubiquitin in the presence of E1 and Ubc4, we found that all three fragments efficiently promoted auto-ubiquitination (Fig. 2 C). Similarly, purified recombinant fragments also ubiquitinated themselves (Fig. 2 D). The smallest construct used in this experiment, glutathione-*S*-transferase (GST)–ChfrF3, has less than 40 amino acids NH_2-terminal to the RF domain and less than 15 amino acids COOH-terminal to the RF domain. We conclude that the RF domain and its adjacent regions are sufficient for the auto-ubiquitination activity of the Chfr protein. Interestingly, kinetic studies indicate that the FHA domain at the NH_2 terminus of the protein actually reduces the ubiquitination activity of the Chfr protein (Fig. 2 D, compare ChfrF1 with ChfrF2), suggesting a potential role of the FHA domain in regulation of the ligase activity.

To confirm the importance of the RF domain in the ligase activity, we constructed two point mutations in the RF domain (Fig. 3 A). The mutated residues, Ile at amino acid 306 and Trp at amino acid 332, are well conserved within a subgroup of RF domains that act as ubiquitin ligases (Fig. 3 A). ChfrI306A and ChfrW332A were expressed in Sf9 cells and purified to homogeneity. When analyzed for auto-ubiquitination activity, both mutants at a low protein concentration (40 μg/ml) failed to auto-ubiquitinate, whereas the wild-type Chfr protein efficiently auto-ubiquitinated (Fig. 3 B). However, the mutant proteins did self-ubiquitinate at a higher protein concentration (200 μg/ml), albeit at a still lower efficiency than the wild-type protein.

We next examined whether these mutant proteins have ubiquitin ligase activity in vivo. Myc-tagged Chfr, ChfrI306A, and ChfrW332A were transfected into HEK293T cells, and all three proteins were expressed at the same level (Fig. 3 C). Myc-tagged proteins were immunopurified by an anti-Myc antibody and then assayed by Western blot analysis with an anti-ubiquitin antibody. The wild-type Chfr immunoprecipitate, but not the mutant Chfr immunoprecipitates, contained ubiquitinated protein(s) (Fig. 3 D). When the Myc–Chfr immunoprecipitates were incubated with radioactive ubiquitin in the presence of E1 and Ubc4, only the wild-type Chfr complex efficiently promoted the formation of ubiquitin conjugates (Fig. 3 E). Neither ChfrI306A nor ChfrW332A were active under physiological conditions, consistent with the biochemical analysis of their ligase activity using recombinant proteins. We conclude that the ubiquitin ligase activity in the Chfr immunoprecipitate results directly from the Chfr protein, and the RF domain in Chfr is essential for the ligase activity in vivo.

The Chfr ligase acts with Ubc4 and Ubc5, but not with UbcH7 and UbcH10

We determined the specificity of the ubiquitin-conjugating enzymes for the Chfr ligase. An immunofluorescence study of the GFP–Chfr fusion protein indicated that Chfr is a nuclear protein (unpublished data). There are several ubiquitin-conjugating enzymes that have been implicated in nuclear functions; these include Ubc4, Ubc5A, Ubc5B, and UbcH10 (Jentsch et al., 1990; Hershko and Ciechanover, 1998). We tested whether these E2s act with Chfr in the ubiquitination reaction. We also included UbcH7 as a negative control in our analysis, because UbcH7 is involved in ubiquitinating ER-associated proteins.

Ubc4, Ubc5A, Ubc5B, UbcH10, and UbcH7 were expressed as recombinant proteins in *E. coli* and purified to homogeneity (unpublished data). When incubated with recombinant E1 and radioactive ubiquitin, all these E2s formed conjugates with ubiquitin that were only detectable by nonreducing SDS-PAGE (Fig. 4 A), suggesting that these conjugates were formed through thioester bonds. Thus, the recombinant E2s analyzed here are all active.

We next examined whether various E2s can promote the formation of Chfr–Ub conjugates. When incubated with recombinant Chfr and radioactive ubiquitin, only Ubc4, Ubc5A, and Ubc5B, not UbcH10 and UbcH7, were active

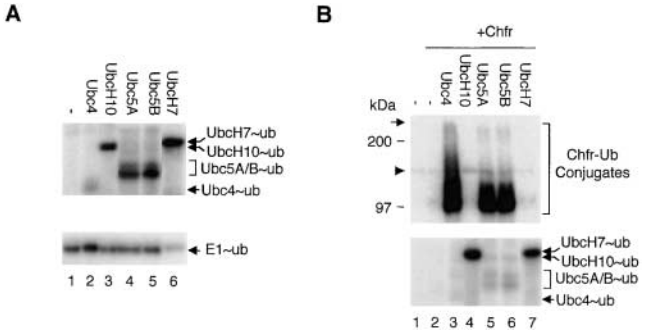

Figure 4. **Chfr functions with Ubc4 and Ubc5, but not with UbcH7 and UbcH10.** (A) A thioester assay for the activity of recombinant ubiquitin-conjugating enzymes. Recombinant E2s were incubated with E1 and then analyzed by nonreducing SDS-PAGE for E1 and E2s conjugated to ubiquitin through a thioester bond. Lane 1, E1 alone. (B) Purified recombinant Chfr (lanes 2–7) was incubated with radioactive ubiquitin in the presence of E1 and various E2s. The formation of the ubiquitin–Chfr conjugates was analyzed by reducing SDS-PAGE (top) and the formation of ubiquitin–E2s conjugates linked through a thioester bond was assayed by nonreducing SDS-PAGE (bottom). Under the reaction conditions, all the Chfr–Ub conjugates entered the separating gel. Arrow indicates the junction between the stacking and separating gels. Arrowhead points to an E1–Ub conjugate that is also present in control lanes (1 and 2). Lane 1, E1 alone. Lane 2, E1 and Chfr.

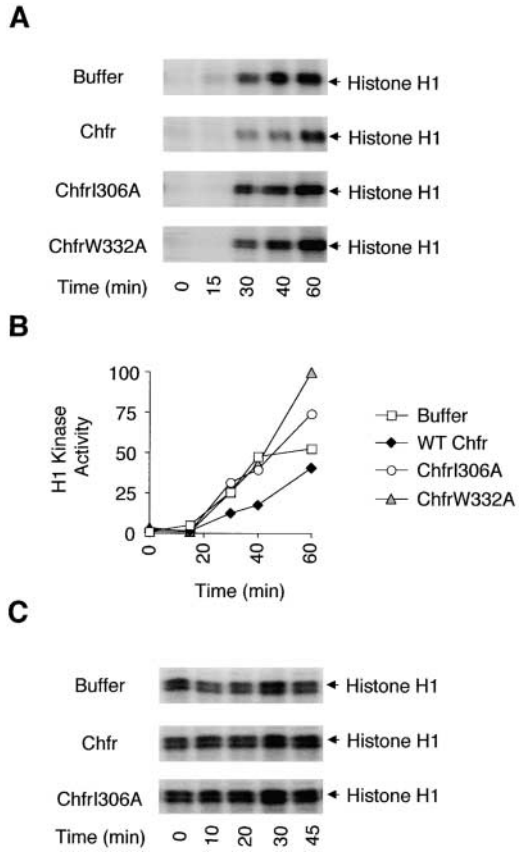

Figure 5. **Chfr delays the activation of the Cdc2 kinase at the G2-M transition.** (A and B) *Xenopus* interphase extracts were incubated with a buffer, Chfr, ChfrI306A, or ChfrW332A. Δ90 cyclin B was then added and the kinetics of the activation of the Cdc2 kinase was analyzed by measuring the phosphorylation of histone H1 with radioactive γ-ATP. The Cdc2 kinase activity was quantitated and plotted against time (B). The unit for kinase activity is arbitrary. In five separate experiments, the wild-type Chfr protein always delayed the activation of Cdc2 kinase. The levels of the Cdc2 activity at 60 min, from extracts treated with ChfrI306A and ChfrW332A, were slightly variable; in some experiments, these levels were close to, instead of higher than, that of the buffer control. (C) *Xenopus* mitotic extracts were incubated with a buffer, Chfr, or ChfrI306A, and the level of the Cdc2 kinase activity was analyzed using histone H1 as a substrate.

in mediating the formation of Chfr–Ub conjugates (Fig. 4 B, top). In the same reactions, both UbcH10–Ub and UbcH7–Ub conjugates accumulated to high levels, as assayed by nonreducing SDS-PAGE (bottom), but ubiquitin failed to transfer from E2 to Chfr.

Chfr inhibits the activation of MPF

Chfr delays entry into mitosis when the checkpoint pathway is activated by mitotic stress, induced with taxol or nocodazole (Scolnick and Halazonetis, 2000). We developed a cell-free system to investigate how the Chfr pathway delays the cell cycle progression.

Entry into mitosis can be monitored in *Xenopus* extracts (Murray and Kirschner, 1989; Solomon et al., 1990). Addition of Δ90 cyclin B, a mutant cyclin B with the first 90 amino acids truncated, to interphase extracts efficiently activated the Cdc2 kinase activity and drove extracts into mitosis (Fig. 5 A, top). However, the addition of wild-type Chfr protein reduced the rate of Cdc2 activation and delayed entry into mitosis (Fig. 5, A and B). This inhibition of Cdc2 kinase activity required an active ligase, because the two inactive mutants, ChfrI306A and ChfrW332A, both failed to delay mitotic entry. Thus, Cdc2 is a key target for the Chfr pathway to control the entry into mitosis.

We then examined whether the Chfr protein has any effect on cells already in mitosis. *Xenopus* mitotic extracts have a high level of Cdc2 kinase activity, which is not affected by the addition of wild-type Chfr or ChfrI306A protein to mitotic extracts (Fig. 5 C). Thus, the Chfr pathway only regulates the initial activation of the Cdc2 kinase at the G2 to M transition.

Chfr is a ubiquitin ligase and addition of an active ligase to extracts could have a nonspecific effect on the entry into mitosis if there is a specific requirement for ubiquitin-dependent proteolysis in the G2 to M transition. For example, active Chfr ligase may nonspecifically interfere with physiological ubiquitin-dependent proteolysis by depleting the ubiquitin pool or by competing for proteasome degradation in extracts. To establish whether there is a requirement for ubiquitin-dependent proteolysis at the G2 to M transition, we examined the effect of methylated ubiquitin (methyl-ubiquitin) and *N*-acetyl-Leu-Leu-norleucinal peptide (LLnL) on entry into mitosis. Methyl-ubiquitin interferes with the formation of poly-ubiquitin conjugates required for degradation and LLnL is an inhibitor of proteasomes. Neither methyl-ubiquitin nor LLnL delayed the mitotic entry, as measured by the kinetics of activation of the Cdc2 kinase (Fig. 6 A, left). The amount of methyl-ubiquitin and LLnL used in these experiments, when added to mitotic extracts,

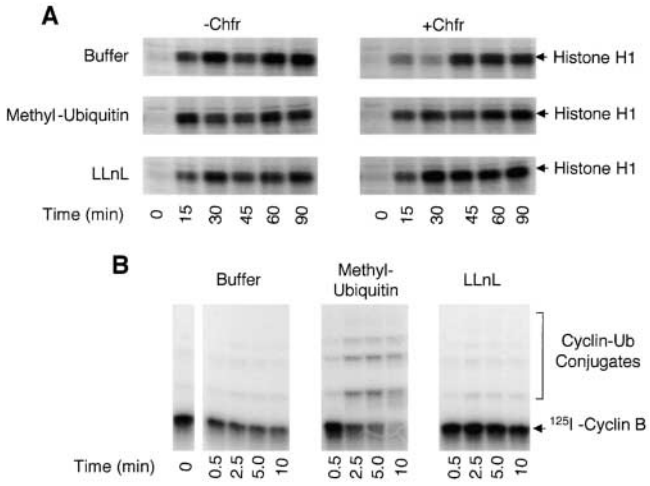

Figure 6. **Chfr-mediated inhibition of Cdc2 requires ubiquitin-dependent protein degradation.** (A) *Xenopus* interphase extracts were incubated with a buffer or Chfr in the presence or absence of methyl-ubiquitin and LLnL. Δ90 cyclin B was then added and the kinetics of the activation of the Cdc2 kinase was analyzed using histone H1 as a substrate. The exact kinetics of activation of Cdc2 in buffer control here are different from that in Fig. 5 due to variations between different extracts. (B) Methyl-ubiquitin and LLnL effectively inhibit ubiquitin-dependent proteolysis. *Xenopus* mitotic extracts were incubated with a buffer, methyl-ubiquitin, or LLnL. Radioactive cyclin B was then added and the kinetics of its degradation was analyzed by SDS-PAGE.

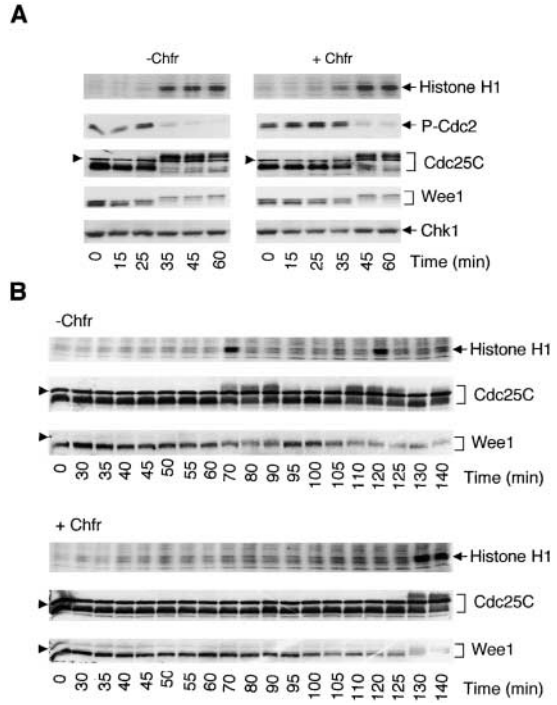

Figure 7. **Chfr prolongs the phosphorylated state of Tyr15 in Cdc2 at the G2-M transition.** (A) *Xenopus* interphase extracts were incubated with a buffer or Chfr. Δ90 cyclin B was then added and the activation of the Cdc2 kinase was analyzed by measuring the phosphorylation of histone H1. The kinetics of entry into mitosis was also measured by the level and the phosphorylation state of Cdc2, Cdc25C, Wee1, and Chk1 in Western blot analysis. Arrowheads point to nonspecific, cross-reacting bands. (B) *Xenopus* cycling extracts were incubated with a buffer or Chfr and the kinetics of entry into mitosis was measured by the Cdc2 kinase activity and by the phosphorylation state of Cdc25C and Wee1. Arrowheads point to nonspecific, cross-reacting bands.

reduced the rate of cyclin degradation (Fig. 6 B), suggesting that both methyl-ubiquitin and LLnL interfere with ubiquitin-dependent proteolysis pathways in *Xenopus* extracts. We conclude that there is no requirement for ubiquitin-dependent proteolysis at the G2 to M transition under normal physiological conditions.

If Chfr ligase activity is required for the delay in mitotic entry, inhibition of ubiquitination or degradation in extracts should abolish the Chfr-dependent delay. This is indeed the case, as addition of either methyl-ubiquitin or LLnL abolished the Chfr-mediated delay before entry into mitosis (Fig. 6 A). We conclude that the Chfr-dependent checkpoint controls entry into mitosis through ubiquitin-dependent proteolysis.

Chfr prolongs the phosphorylated state of Tyr15 in Cdc2 at the G2 to M transition

We investigated how the Cdc2 kinase activity is regulated by Chfr. When recombinant Chfr was incubated with interphase extracts and Δ90 cyclin B, the Chfr pathway delayed the removal of inhibitory phosphorylation on Cdc2, as analyzed by an antibody specific to the tyrosine 15–phosphorylated Cdc2 (Fig. 7 A). 25 min after addition of Δ90 cyclin B, Cdc2 from both the control extracts and the Chfr-treated extracts had significant levels of phosphorylated tyrosine. Interestingly, by 35 min, the level of phosphorylated tyrosine was greatly reduced in control extracts, correlating well with the activation of the Cdc2 kinase (Fig. 7 A). On the other hand, the level of phosphorylated tyrosine in Chfr-treated extracts remained very high, consistent with the observation that the Cdc2 kinase was not activated until 45 min after the addition of Δ90 cyclin B (Fig. 7 A). We conclude that Chfr delays mitotic entry by prolonging the phosphorylated state of Cdc2.

At the G2 to M transition, Cdc2 is regulated by the Wee1 kinase through inhibitory phosphorylation at Thr14 and Tyr15, and by the Cdc25C phosphatase through dephosphorylation (King et al., 1994). Wee1 and Cdc25C are regulated posttranslationally. Phosphorylation of Cdc25C activates its phosphatase activity, whereas phosphorylation of Wee1 inactivates its kinase activity (Izumi et al., 1992; Kumagai and Dunphy, 1992, 1996; Izumi and Maller, 1993). We found that Chfr delayed phosphorylation of both Wee1 and Cdc25C at the G2 to M transition (Fig. 7 A). On the other hand, Chfr had no effect on the steady-state level of Cdc2, Wee1, and Cdc25C proteins, as extracts entered mitosis (Fig. 7 A; and unpublished data), suggesting that not one of these three proteins is the direct target for ubiquitination by Chfr. Similarly, the level of Chk1, a regulator of Cdc25C at the G2 to M transition (Peng et al., 1997; Sanchez et al., 1997), was not affected by Chfr (Fig. 7 A). We conclude that the Chfr-mediated delay on the activation of the Cdc2 kinase is due to a combination of over-activa-

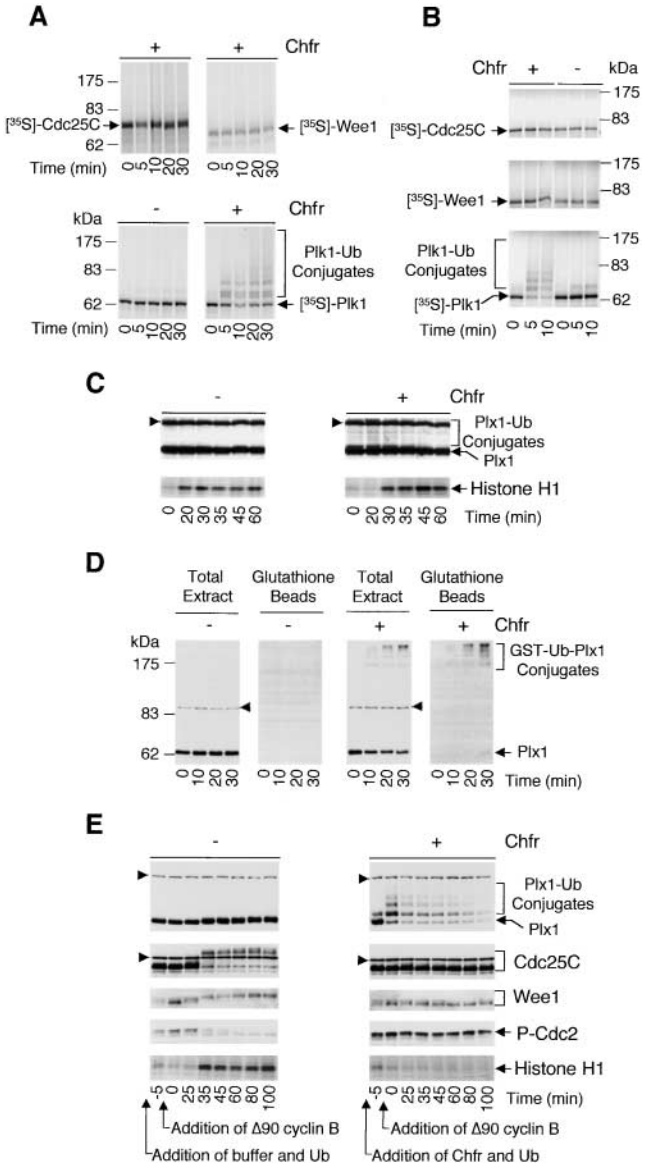

Figure 8. **Plk1 is the substrate of Chfr ligase.** (A) In vitro–translated Cdc25, Wee1, and Plk1 were incubated with *Xenopus* interphase extracts and ubiquitin, either in the presence or absence of recombinant Chfr, and the ubiquitination of these proteins was analyzed by SDS-PAGE. (B) In vitro–translated Cdc25, Wee1, and Plk1 were incubated with recombinant E1, E2, ubiquitin, and ATP, either in the presence or absence of recombinant Chfr, and the ubiquitination of these proteins was analyzed by SDS-PAGE. (C) *Xenopus* interphase extracts were incubated with a buffer or Chfr. Δ90 cyclin B was then added and the kinetics of the activation of the Cdc2 kinase was analyzed by measuring the phosphorylation of histone H1. Duplicated samples were analyzed in Western blot with an antibody against *Xenopus* Plx1. Arrowheads point to nonspecific, cross-reacting bands. (D) *Xenopus* interphase extracts were incubated with GST–Ub plus a buffer or Chfr. Samples were collected at various time points to directly blot against an anti-Plx1 antibody. In addition, GST–Ub conjugates were purified with glutathione beads and then assayed by Western blot analysis using the anti-Plx1 antibody. Arrowheads point to nonspecific, cross-reacting bands. (E) *Xenopus* interphase extracts were incubated with recombinant ubiquitin (at 2 mg/ml) plus a buffer or Chfr for 5 min. Δ90 cyclin B was then added and the kinetics of entry into mitosis was measured by the Cdc2 kinase activity and by the phosphorylation state of Cdc2, Cdc25C, and Wee1. The degree of ubiquitination of Plx1 was assayed by Western blotting with an anti-Plx1 antibody. Arrowheads point to nonspecific, cross-reacting bands.

tion of the inhibitory kinase Wee1 and an inhibition of the Cdc25C phosphatase.

We observed a more pronounced delay of mitotic entry in *Xenopus* cycling extracts. Control extracts entered mitosis twice in the first 2 h, first at 70 min and then at 120 min, as analyzed by the activity of the Cdc2 kinase and the phosphorylation states of Cdc25C and Wee1 (Fig. 7 B). However, the entry into mitosis in Chfr-treated extracts was delayed by more than one cell cycle; such extracts did not enter mitosis until 130 min. This delay for the G2 to M transition correlates well with the delayed phosphorylation of Cdc25C and Wee1 (Fig. 7 B). It is likely that the shorter delay observed in extracts treated with Δ90 cyclin B reflects the high concentration of Δ90 cyclin B used in these experiments, and that cycling extracts more closely reflect the physiological process in vivo.

Chfr ubiquitinates Polo-like kinase 1 (Plk1)

To identify the substrate of the Chfr ligases, we analyzed the ability of Chfr to ubiquitinate regulators of Cdc2 in *Xenopus* extracts. The addition of recombinant Chfr to interphase extracts did not change the stability of in vitro translated Cdc25C and Wee1 (Fig. 8 A), consistent with the Western blot analysis of the endogenous Cdc25C and Wee1 (Fig. 7). Thus, neither Cdc25C nor Wee1 is the substrate of Chfr. We next tested the stability of Plk1, a positive regulator for Cdc25C and a negative regulator for Wee1 at the G2 to M transition (King et al., 1994). When incubated with interphase extracts, in vitro–translated Plk1 was efficiently ubiquitinated in a Chfr-dependent manner (Fig. 8 A). Indeed, Plk1 is a substrate of Chfr. In an in vitro reconstituted reaction, Plk1, but not Cdc25C and Wee1, was quantitatively ubiquitinated in the presence of purified recombinant E1, Ubc4, and Chfr (Fig. 8 B).

The endogenous Plx1, the *Xenopus* homologue of human Plk1, was also modified in a Chfr-dependent manner (Fig. 8, C and D). When incubated with Δ90 cyclin B, Chfr delayed entry into mitosis. This delay correlates with the generation of a ladder of Plx1 bands migrating slower than the unmodified Plx1, as analyzed by an anti-Plx1 blotting (Fig. 8 C). To confirm that these slow-migrating bands represented ubiquitinated Plx1, interphase extracts were incubated with GST–Ub in the presence or absence of recombinant Chfr. After various incubation times, two sets of aliquots were collected, one for Western blot analysis by the anti-Plx1 antibody, and the other for purification of GST–Ub conjugates using glutathione agarose beads (Fig. 8 D). Purified conjugates were then analyzed by Western blotting using the anti-Plx1 antibody to detect ubiquitinated Plx1. In the absence of Chfr, Plx1 migrated as a single band and therefore was not ubiquitinated in interphase extracts. Addition of Chfr promoted the formation of high molecular weight forms of Plx1, which were covalently linked to GST–Ub (Fig. 8 D). Thus, endogenous Plx1 is ubiquitinated by Chfr.

The extent of ubiquitination of endogenous Plx1 is limited in interphase extracts. We noticed that *Xenopus* extracts had a limited capacity for ubiquitination and that efficient ubiquitination of cell cycle regulators required exogenously added recombinant ubiquitin protein. For example, com-

plete degradation of mitotic cyclin B by the APC pathway requires the addition of ubiquitin to mitotic extracts (Fang et al., 1998a,b). In the presence of recombinant ubiquitin, endogenous Plx1 was quantitatively ubiquitinated by Chfr and subsequently degraded by proteasomes (Fig. 8 E). Complete ubiquitination and degradation of Plx1 prevented the phosphorylation of Cdc25C and Wee1 in Chfr-treated extracts, such that Cdc2 remained phosphorylated and its kinase activity was not activated, even after extended incubation with Δ90 cyclin B (Fig. 8 E). In the presence of Chfr, even the residual level of Cdc2 kinase activity detectable in interphase extracts was downregulated. We conclude that Chfr ubiquitinates and downregulates Plx1, which, in turn, simultaneously regulates Cdc25C and Wee1 activities, leading to prolonged phosphorylation of Cdc2 and a delay in entry into mitosis.

Discussion

Chfr is a checkpoint protein required for a delay in entry into mitosis in response to mitotic stress (Scolnick and Halazonetis, 2000). We show here that Chfr is a ubiquitin ligase that regulates the activation of the Cdc2 kinase at the G2 to M transition. Chfr targets Plk1 for ubiquitination and degradation, leading to the downregulation of Cdc25C phosphatase activity and upregulation of Wee1 kinase activity. Thus, Chfr defines a novel ubiquitin-dependent proteolysis pathway that controls entry into mitosis.

The checkpoint protein Chfr is a ubiquitin ligase

Chfr is a ubiquitin ligase based on the following observations. First, the Chfr protein has a ring finger domain common to a growing number of ubiquitin ligases (Jackson et al., 2000; Joazeiro and Weissman, 2000; Scolnick and Halazonetis, 2000). Sequence alignment of the Chfr ring finger domain with those from APC11 and c-Cbl indicates that they are highly homologous. The cocrystal structure of c-Cbl and UbcH7 have been solved recently and the ring finger domain in c-Cbl has been shown to directly bind to UbcH7 (Zheng et al., 2000), indicating an important role of the ring finger domain in the ubiquitin ligase activity. Residues in c-Cbl involved in interacting with UbcH7 are partly conserved in Chfr; these residues include Ile306, Cys328, Trp332, and Pro340 (amino acid numbers derived from the Chfr protein). Second, recombinant Chfr auto-ubiquitinates in an E1- and E2-dependent manner. Auto-ubiquitination is a hallmark of ring finger–containing ligases (Joazeiro et al., 1999; Huang et al., 2000; Suzuki et al., 2001). Indeed, both c-Cbl and APC11 have auto-ubiquitination activities in the absence of substrates (Joazeiro et al., 1999; Gmachl et al., 2000; Leverson et al., 2000). We showed here that the ring finger domain in Chfr is both necessary and sufficient for the auto-ubiquitination activity. Third, when expressed in mammalian tissue culture cells, the Chfr immunoprecipitate also contains a ubiquitinated protein(s) and promotes the formation of high molecular weight ubiquitin conjugates, indicating that the Chfr immunoprecipitate has a ubiquitin ligase activity. Again, the ring finger domain in Chfr is essential for the ligase activity in vivo, because point mutations of conserved residues in this domain abolish the ubiquitin ligase activity. Fourth, Plk1 is ubiquitinated in *Xenopus* extracts in a Chfr-dependent manner, and in a purified, reconstituted system, Chfr directly ubiquitinates Plk1. Thus, Chfr by itself is sufficient to ubiquitinate the substrate.

How is the ligase activity toward itself and the substrates differentially regulated? There are at least two possible mechanisms for this regulation. First, the auto-ubiquitination activity may be separable from the ligase activity toward substrates, with self-ubiquitination and ubiquitination of substrates being mutually exclusive. An example of a ligase with such a mechanism is the MDM2 protein, a ring finger–containing ligase for the tumor suppressor p53. MDM2 ubiquitinates itself as well as p53 (Fang et al., 2000). The ligase is modified in the ring finger domain by SUMO-1, a ubiquitin-like protein. Modification by SUMO-1 prevents self-ubiquitination and increases its ligase activity toward p53 (Buschmann et al., 2000). On the other hand, a reduction of the SUMO-1 modification destabilizes MDM2 and increases the level of p53. A second possibility is that Chfr may ubiquitinate itself and its substrates at the same time, and ubiquitinated Chfr and substrates are degraded by proteasomes. Such a mechanism exists for the c-Cbl ligase. C-Cbl mediates the ubiquitination of itself and c-Src. Ubiquitination of both proteins requires Src kinase activity and tyrosine phosphorylation of c-Cbl by Src (Yokouchi et al., 2001), suggesting that substrate ubiquitination and auto-ubiquitination are interdependent. The *Xenopus* extract system should allow us to determine the regulatory mechanism of Chfr self-ubiquitination versus ubiquitination of Plk1.

Chfr delays entry into mitosis by inhibiting the activation of Cdc2

The Chfr-mediated checkpoint controls entry into mitosis (Scolnick and Halazonetis, 2000). Normal primary cells and tumor cell lines with wild-type Chfr exhibit a delay in entry into mitosis when centrosome separation is inhibited by nocodazole or taxol. On the other hand, tumor cell lines that lack the functional Chfr protein enter mitosis without such a delay in the presence of mitotic stress. Importantly, ectopic expression of Chfr in tumor cell lines restores the delay, indicating that the Chfr protein is responsible for the delay in entry into mitosis. Similarly, ectopic expression of the active Chfr protein in *Xenopus* extracts is likely to mimic a checkpoint-activated state, and our cell-free system allows us to investigate the molecular pathway that Chfr regulates. It is interesting to note that the Chfr-minus cells seem to have a normal G2 to M transition in the absence of mitotic stress (Scolnick and Halazonetis, 2000). Thus, the Chfr protein is only required for a checkpoint mechanism that controls the kinetics of mitotic entry in the presence of mitotic stress. This is consistent with our observation that there is no requirement for ubiquitin-dependent proteolysis at the normal G2 to M transition. Although we have not examined the function of endogenous Chfr protein in *Xenopus* extracts due to the lack of a high-quality anti-Chfr antibody for immunodepletion experiments, the endogenous Chfr, if expressed in *Xenopus* embryonic extracts, is unlikely to be required for entry into mitosis in the absence of the checkpoint signal.

When added to *Xenopus* extracts, the Chfr ligase delays entry into mitosis with a prolonged phosphorylation of tyrosine 15 in the Cdc2 kinase. In cycling extracts, the Chfr protein delays the activation of Cdc2 by more than one cell cycle. Given that the inactive Chfr mutants have no effect on mitotic entry, and the inhibition of ubiquitin-dependent protein degradation rescues the delay phenotype, the effect of Chfr on mitotic entry is specific and requires ubiquitin-dependent protein degradation.

It is interesting to note that in mammalian tissue culture cells, the Chfr pathway causes a delay in entry into mitosis without a detectable delay in the activation of the Cdc2 kinase (Scolnick and Halazonetis, 2000). It is possible that the temporal delay in activation of Cdc2 kinase found in *Xenopus* extracts could have escaped from detection in the mammalian system due to a large time interval (2 h) between time points used in the original analysis of the Cdc2 kinase activity. This discrepancy between *Xenopus* extracts and mammalian tissue culture cells may also result from difficulties in achieving an optimal synchronization in mammalian cells (Scolnick and Halazonetis, 2000). In addition, we found that constitutive expression of Chfr in mammalian cells is detrimental to cell proliferation and the expression level of the exogenously introduced Chfr was quickly diminished in stable Chfr cell lines (unpublished data). On the other hand, it has been reported that the Chfr checkpoint temporarily arrests mammalian cells at prophase with an intact nuclear envelope and uncondensed chromosomes (Scolnick and Halazonetis, 2000), consistent with a low level of the Cdc2 kinase activity.

Plk1 is ubiquitinated by Chfr

The direct target of the Chfr pathway is Plk1. In vitro–translated Plk1 is ubiquitinated, in a Chfr-dependent manner, both in *Xenopus* interphase extracts as well as in a purified system reconstituted with recombinant proteins. On the other hand, neither Cdc25C nor Wee1 is ubiquitinated in both assays, demonstrating the specificity of the Chfr pathway. The endogenous Plx1 is also ubiquitinated in Chfr-treated *Xenopus* extracts, and this ubiquitination correlates with the delay in entry into mitosis. Indeed, if the efficiency of the Chfr pathway is augmented by recombinant ubiquitin, endogenous Plx1 is quantitatively ubiquitinated and the Chfr-treated extracts are arrested at G2 with minimal Cdc2 kinase activity. We expect that *Xenopus* extracts reflect certain aspects of Plk1 regulation in mammalian somatic cells. The expression of Plk1 is regulated in mammalian cells; Plk1 begins to accumulate in G2 and its level peaks in mitosis (Fang et al., 1998a). Activation of the Chfr pathway by mitotic stress leads to ubiquitination and degradation of Plk1, and blocks entry into mitosis. Once mitotic stress is relieved and defects are repaired, the Chfr ligase is inactivated and de novo synthesis of Plk1 drives cells into mitosis.

Plk1 regulates both the Wee1 kinase and the Cdc25C phosphatase, which in turn control the Cdc2 kinase activity at the G2 to M transition (Russell and Nurse, 1986; Gould and Nurse, 1989; Ferrell et al., 1991; Gautier et al., 1991; Kumagai and Dunphy, 1991; Osmani et al., 1991; Kumagai and Dunphy, 1992). Wee1 mediates the inhibitory phosphorylation of Tyr15 and Thr14 on Cdc2, whereas Cdc25C dephosphorylates and activates Cdc2. Cdc25C and Wee1 themselves are regulated by complex molecular circuits in the cell cycle (King et al., 1994). The NH_2-terminal regulatory domain in Cdc25C is phosphorylated by Plk1 and Cdc2/cyclin B, and phosphorylation enhances the phosphatase activity of Cdc25C (Izumi et al., 1992; Kumagai and Dunphy, 1992; Izumi and Maller, 1993; Kumagai and Dunphy, 1996). On the other hand, Wee1 is also likely to be phosphorylated by Plk1 and Cdc2/cyclin B, but phosphorylation inhibits the kinase activity of Wee1. Thus, the activation of Cdc25C and Cdc2 and the inhibition of Wee1 at mitosis constitute an auto-activating feedback loop that allows the Cdc2 kinase to be activated in an all-or-none fashion (King et al., 1994). The initial activity of Plk1 at the G2 to M transition can determine the status of the auto-activating loop and the level of the Cdc2 kinase activity.

We reported here that Plk1 is a target for the Chfr-mediated checkpoint control. Plk1 is a kinase initially identified for its role in controlling the separation of duplicated centrosomes at the G2 to M transition (Llamazares et al., 1991; Lane and Nigg, 1996). Thus, the Chfr pathway may also delay the separation of centrosomes in the presence of microtubule drugs, thereby providing additional time for repairing defects in the centrosome structure. We speculate that the Chfr checkpoint may control multiple steps at the G2 to M transition. Indeed, in the two other ubiquitination pathways, SCF ligase and APC ligase, each has multiple substrates and controls several events in the cell cycle (Fang et al., 1999; Jackson et al., 2000). The demonstration of Chfr as a ubiquitin ligase and the identification of Plk1 and Cdc2 as targets for the Chfr pathway will allow us to begin to understand the biological function of this novel ubiquitination pathway.

Materials and methods

Antibodies

Rabbit and mouse anti-Myc antibodies, anti-ubiquitin antibody, and anti-Cdc2 antibody were purchased from Santa Cruz Biotechnology Inc., and were used at 0.1 μg/ml for immunoprecipitation and at 1 μg/ml for Western blot analysis. Rabbit antibody against Tyr15-phosphorylated Cdc2 was purchased from Cell Signaling, Inc., and the mouse anti-Plx1 antibody was from Zymed Laboratories. Antibodies against *Xenopus* cyclin A, Wee1, and Cdc25C were gifts from Drs. Tod Stukenberg (University of Virginia, Charlottesville, VA), Bill Dunphy (California Institute of Technology, Pasadena, CA), Jim Ferrell (Stanford University, Palo Alto, CA), and James Maller (University of Colorado, Denver, CO).

Expression and purification of recombinant proteins

Human Chfr gene was subcloned by PCR amplification into pFastBac (GIBCO BRL), expressed as a soluble protein in Sf9 cells, and purified to homogeneity. ChfrI306A and ChfrW332A mutants were constructed with the Quick Change Site-Directed Mutagenesis Kit (Stratagene), and mutant proteins were expressed and purified. Deletion mutants of Chfr were constructed by PCR amplification and mutant proteins were expressed in *E. coli*. ChfrF1 and ChfrF2 were purified by Ni-NTA beads (QIAGEN), and GST–ChfrF3 was purified by glutathione-agarose beads (Amersham Pharmacia Biotech). Human Ubc5A, Ubc5B, and UbcH7 genes were cloned into a modified pET28a vector by PCR amplification from human fetal thymus cDNA. Recombinant proteins were expressed in *E. coli* and purified by Ni-NTA beads. Expression and purification of recombinant E1, Ubc4, and UbcH10 were described previously (Fang et al., 1998a,b).

Ubiquitination Assays

Ubiquitin (Sigma-Aldrich) was labeled with ^{125}I to a specific activity of 100 μCi/μg using the chloramine T procedure. For auto-ubiquitination of recombinant Chfr in Figs. 1–3, reactions were performed in a total volume of

10 μl. The reaction mixture contained an energy-regenerating system, 400 μg/ml labeled ubiquitin, 20 μg/ml recombinant E1, 10 μg/ml Ubc4, and various amounts of Chfr as indicated in figure legends. Reactions were incubated at room temperature for various times, quenched with SDS sample buffer, and analyzed by reducing 12% SDS-PAGE. Gels were scanned with a PhosphorImager (Molecular Dynamics).

For ubiquitination assays with Myc–Chfr complexes from HEK293T cells (Figs. 1 and 3), anti-Myc immunoprecipitates were purified from one jumbo dish of transfected HEK293T cells and incubated with 400 μg/ml labeled ubiquitin, 20 μg/ml recombinant E1, 300 μg/ml Ubc4, and an energy-regenerating system at room temperature for 1 h. The reactions were quenched with SDS sample buffer and analyzed by reducing 10% SDS-PAGE.

For auto-ubiquitination assays with various E2s in Fig. 4 B, Chfr at 400 μg/ml were incubated at room temperature for 5 min with 30 μg/ml labeled ubiquitin, 200 μg/ml recombinant E1, 200 μg/ml various E2s, and an energy-regenerating system. The reactions were quenched with SDS sample buffer and analyzed by reducing 10% SDS-PAGE.

For ubiquitination of Plk1, recombinant Chfr was incubated at room temperature for various times with in vitro–translated [^{35}S]Plk1 in the presence of E1, Ubc4, ubiquitin, and ubiquitin aldehyde, quenched with SDS sample buffer, and analyzed by SDS-PAGE. To confirm that the endogenous Plx1 is ubiquitinated in a Chfr-dependent manner, immobilized GST–Ub was incubated with *Xenopus* interphase extracts in the presence or absence of recombinant Chfr protein. The final concentration of GST–Ub in extracts was 0.85 mg/ml. Ubiquitinated products were purified by glutathione beads and immunoblotted with an anti-Plx1 antibody.

The thioester assay for E2, histone H1 kinase assay, and cyclin degradation assay were performed as described previously (Fang et al., 1998a,b).

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