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Cyclin F Is Degraded during G_2-M by Mechanisms Fundamentally Different from Other Cyclins*

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Cyclin F, a cyclin that can form SCF complexes and bind to cyclin B, oscillates in the cell cycle with a pattern similar to cyclin A and cyclin B. Ectopic expression of cyclin F arrests the cell cycle in G_2/M. How the level of cyclin F is regulated during the cell cycle is completely obscure. Here we show that, similar to cyclin A, cyclin F is degraded when the spindle assembly checkpoint is activated and accumulates when the DNA damage checkpoint is activated. Cyclin F is a very unstable protein throughout much of the cell cycle. Unlike other cyclins, degradation of cyclin F is independent of ubiquitination and proteasome-mediated pathways. Interestingly, proteolysis of cyclin F is likely to involve metalloproteases. Rapid destruction of cyclin F does not require the N-terminal F-box motif but requires the COOH-terminal PEST sequences. The PEST region alone is sufficient to interfere with the degradation of cyclin F and confer instability when fused to cyclin A. These data show that although cyclin F is degraded at similar time as the mitotic cyclins, the underlying mechanisms are entirely distinct.

Cyclins and cyclin-dependent kinases (CDKs)[1] are key regulators of the eukaryotic cell cycle. In mammalian cells, different cyclin-CDK complexes are involved in regulating different cell cycle transitions: cyclin D-CDK4/6 for G_1 progression, cyclin E-CDK2 for the G_1-S transition, cyclin A-CDK2 for S phase progression, and cyclin A/B-CDC2 for entry into M phase (1). Apart from these well known roles in the cell cycle, several cyclins and CDKs are involved in processes not directly related to the cell cycle. Cyclin D can bind and activate the estrogen receptor, and CDK5 is activated in postmitotic neurons by p35. The cyclin H-CDK7 complex is a component of both the CDK-activating kinase and the basal transcription factor TFIIH and can phosphorylate CDKs and the carboxyl-terminal repeat domain of the large subunit of RNA polymerase II, respectively. Other cyclins and CDKs (cyclin C-CDK8, cyclin T-CDK9, and cyclin K) are also known to associate with RNA polymerase II and phosphorylate the carboxyl-terminal repeat domain. Cyclin G, a target of p53, recruits PP2A to dephosphorylate MDM2 (2). Finally, cyclin T-CDK9 interacts directly with the human immunodeficiency virus type-1 Tat protein and enhances Tat binding to the viral TAR RNA stem-loop structure.

Despite the large amount of information known about cyclins, the functions and regulation of several "orphan" cyclins, notably cyclin F and cyclin I, remain to be determined. Cyclin F is the largest and in many respects one of the most interesting members of the cyclin family. Apart from the cyclin box region common to all cyclins, cyclin F also contains an F-box motif at the N terminus and PEST sequences at the COOH-terminal quarter of the protein. The F-box motif (which originally derived its name from cyclin F) is responsible for binding to SKP1 in the formation of the SCF ubiquitin ligase complex (3). The PEST sequence (proline-, glutamic acid-, serine-, and threonine-rich) is typically present in rapidly turnover proteins (4). Finally, the cyclin box of cyclin F is highly unusual, because no CDK partners have been identified, and it can bind to another molecule of cyclin (5).

The expression profile of cyclin F is most similar to that of cyclin A. Cyclin F mRNA begins to accumulate in S phase, peaks in G_2, and declines before mitosis. The accumulation and decline of cyclin F mRNA occur slightly ahead of cyclin B1 and nearly coincide with the profile of cyclin A mRNA (6). Accumulation of cyclin A and cyclin F mRNA during G_2 phase has been confirmed by microarray analysis (7). At the protein level, cyclin F accumulates during S phase and decreases around mitosis, again similar to cyclin A (6, 8).

Overexpression of cyclin F causes an accumulation of the G_2/M population (6). This effect is stronger with a mutant cyclin F lacking the PEST region. However, it is not clear whether this is due to a delay of G_2/M, a shortening of G_1 or S, or the competition with a common CDK partner. Overexpressed cyclin F accumulates in the nucleus due to the presence of two nuclear localization sequences, but a portion of cells also displays perinuclear staining (5, 6). In this connection, it has been shown that the cyclin box region of cyclin F can bind to the cytoplasmic retention sequence region of cyclin B1 by yeast two-hybrid, *in vitro* binding, and co-immunoprecipitation assays (5). It is believed that during the G_2/M transition, cyclin F may carry cyclin B1 (which lacks its own nuclear localization sequence) into the nucleus by virtue of the nuclear localization sequence in cyclin F.

Ubiquitin/proteasome-dependent proteolysis of cyclin A and cyclin B requires a short sequence near their N terminus called the destruction box (D-box) (9, 10). The ubiquitin ligase E3 for cyclin B1 in mitosis is the anaphase-promoting complex/cyclosome complexed with CDC20 (11). Cyclin A is probably also degraded by anaphase-promoting complex/cyclosome-CDC20 (12–15), although the difference in the timing of degradation between cyclin A and cyclin B1 argues for the involvement of distinct mechanisms.

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[1] The abbreviations used are: CDK, cyclin-dependent kinase; HA, hemagglutinin; ADR, adriamycin; CMP, camptothecin; Ub, ubiquitin.

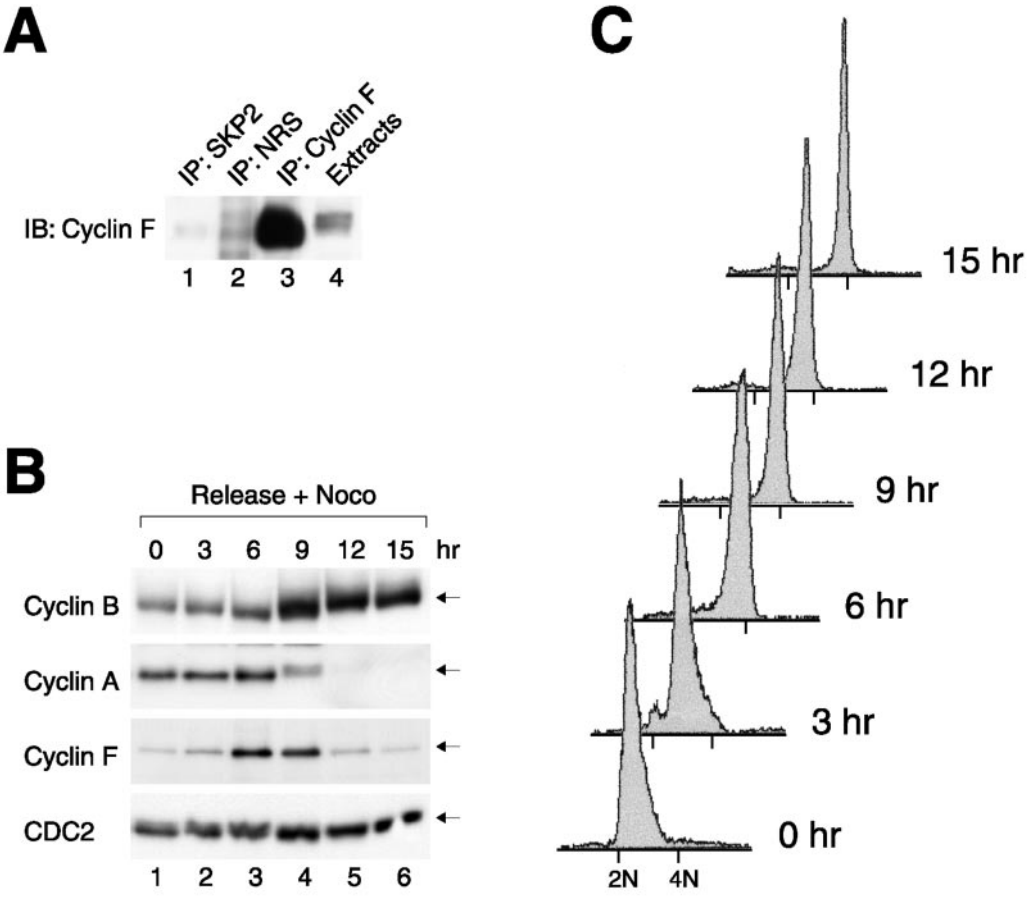

FIG. 1. **Cyclin F is accumulated and destroyed at similar time as cyclin A and is not stabilized by nocodazole.** *A*, anti-cyclin F antibodies. Cell-free extracts (200 μg) were immunoprecipitated with normal rabbit serum (*lane 2*) or antiserum raised against SKP2 (*lane 1*) and cyclin F (*lane 3*). One-third of the immunoprecipitates and 10 μg of total cell lysates (*lane 4*) were loaded onto SDS-PAGE and immunoblotted with antibodies against cyclin F. *B*, cells were synchronously released from early S phase with a double thymidine block method as described under "Experimental Procedures." The cells were prevented to progress through the subsequent mitosis with nocodazole. Cell extracts were prepared at the indicated time points and cyclin B, cyclin A, cyclin F, and CDC2 were detected by immunoblotting. *C*, a portion of cells from the synchronization experiment shown in *B* were stained with propidium iodide and analyzed with flow cytometry. The positions of 2 and 4 N DNA content are indicated.

Despite the fact that cyclin A and cyclin F are degraded at the same time, no D-box sequence is recognized in cyclin F. Instead, PEST sequences at the COOH-terminal quarter of cyclin F may represent a possible destruction signal (4). It is noteworthy that other mammalian cyclins including cyclin D1, cyclin T1, and cyclin G2 also contain PEST sequences. The molecular basis of proteolysis of PEST-containing protein is far from clear. Proteolysis of several PEST-containing proteins involves the Ca^{2+}-dependent protease calpain (16, 17), whereas proteolysis of other PEST-containing proteins by calpain is shown to be independent of the PEST sequences (18, 19).

Another major class of ubiquitin ligase is the SCF complex comprising SKP1, CUL1, and F-box proteins (20). The F-box motif (first found in cyclin F, Cdc4p, and Skp2p) is involved in direct interaction with SKP1 (3). It is believed that SCF complexes composed of different F-box subunits target different substrates for ubiquitination. Examples of SCF functions include the ubiquitination of cyclin E by SCF^{CDC4} and SCF^{FBW7} (21, 22) and $p27^{KIP1}$ by SCF^{SKP2} (22–24). Although cyclin F is one of the founding members of the F-box protein family, it is still unclear whether cyclin F can function as an ubiquitin ligase. Furthermore, little is known about how the variation of cyclin F is controlled in the cell cycle and how it is related to cyclin A and cyclin B. Several F-box proteins including Cdc4p (25–27) and SKP2 (28, 29) are themselves degraded by the ubiquitin/proteasome system, but it is unclear whether the same pathway also degrades cyclin F.

EXPERIMENTAL PROCEDURES

Materials—All reagents were from Sigma unless stated otherwise.

DNA Constructs—Cyclins in this study were of human origin, and the subtypes were cyclin A2, cyclin B1, and cyclin E1. Cyclin F in pET11d, FLAG-cyclin F in pUHD-P1, FLAG-cyclin A in pUHD-P1, and cyclin E in pLINX were as described previously (30). Cyclin F lacking the N-terminal F-box region (NΔ79) or cyclin F with only the PEST region (NΔ565) were constructed by PCR using the primers T7 terminator and 5′-TGCCATGGTCCAGGAGCTGTGGCCGTC-3′ (for NΔ79) or 5′-TCCCATGGGGCGGAGAACCAAAC-3′ (for NΔ565) from cyclin F in pET11d; the *Nco*I-*Eco*RI fragments were then put into pUHD-P1 or pET21d. Cyclin F in pET11d cut with *Ava*I was used directly in *in vitro* translation to produce cyclin F lacking the COOH-terminal PEST region (CΔ565). FLAG-cyclin A-cyclin F(NΔ565) (cyclin A fused to the PEST region of cyclin F) was created by putting the *Nco*I-cut fragment of cyclin A generated from PCR into FLAG-cyclin F(NΔ565) in pUHD-P1. FLAG-SKP2 for mammalian expression and cyclin A and cyclin A(NΔ71) (ΔD-box) for reticulocyte lysate expression were as described previously (31). Human ubiquitin cDNA was a gift from Dr. Tim Hunt (Cancer Research UK). Ubiquitin (Ub) was amplified by PCR with primers 5′-TCCCATGGAAATCTTTGTAAAA-3′ (ubiquitin forward primer introducing an *Nco*I site) and 5′-GGGCCATGGCTAGTTATTGCTCAGCGGTGG-3′ (T7 terminator primer introducing an *Nco*I site), cut with *Nco*I, and ligated into pUHD-P2 (30) to produce hemagglutinin (HA)-Ub in pUHD-P2.

Cell Culture—HtTA1 cells are HeLa cells (human cervical carcinoma) expressing the tTA tetracycline repressor chimera (30). Cells

were blocked at mitosis by incubation in medium containing 0.1 μg/ml nocodazole for 16 h. Cells in G_1 phase were obtained 3 h after released from nocodazole block. Cells were blocked in S phase by incubation in medium containing 1.5 mM hydroxyurea for 24 h. In promoter turn-off experiments, doxycycline (1 μg/ml) was added to the medium at 24 h after transfection, and the cells were harvested at the indicated time. In some experiments, cells were treated with actinomycin D (100 ng/ml), cycloheximide (10 μg/ml), LLnL (also called MG101) (50 μM), and LLM (50 μM) for the indicated time.

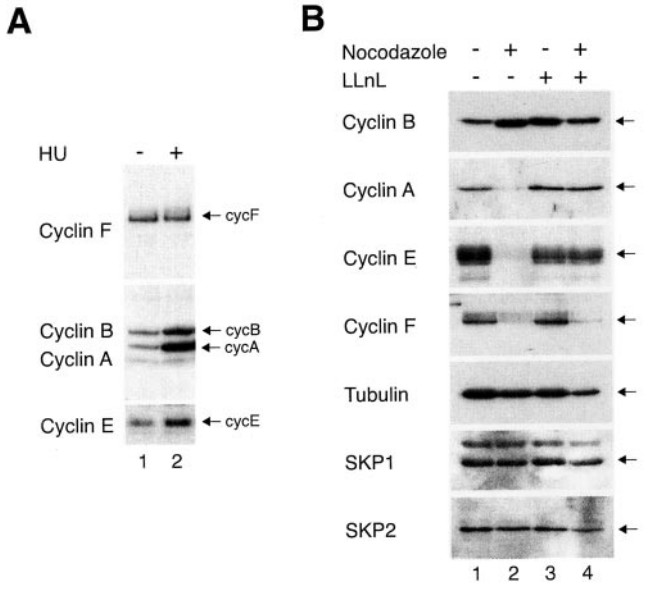

FIG. 2. **Cyclin F is destroyed in nocodazole-treated cells by proteasome-independent mechanisms.** *A*, accumulation of A-, B-, and E-type cyclins but not cyclin F in hydroxyurea-treated cells. HeLa cells were either mock-treated or were blocked in S phase with hydroxyurea as described under "Experimental Procedures." Cell-free extracts were prepared, and cyclin F, cyclin A, cyclin B, and cyclin E were detected by immunoblotting. *B*, accumulation of cyclin B and loss of cyclin A, cyclin E, and cyclin F in nocodazole-treated cells. HeLa cells were either mock-treated (*lanes 1* and *3*) or blocked in metaphase with nocodazole as described under "Experimental Procedures." Proteasome inhibitor LLnL was added 6 h before the cells were harvested. Cell-free extracts were prepared and cyclin B, cyclin A, cyclin E, and cyclin F were detected by immunoblotting. Tubulin and SCF components (SKP1 and SKP2) were also analyzed in the same samples.

Synchronization and Flow Cytometry—Cells were synchronized with a double thymidine method as described previously (8). Nocodazole (0.1 mg/ml) was added when the cells were released from the second block to prevent them from progressing through the subsequent mitosis. Flow cytometry after propidium iodide staining was performed as described previously (32).

Expression of Recombinant Proteins—Transient transfection and preparation of cell-free extracts were performed as described previously (30). Coupled transcription-translation reactions in the presence of [^{35}S]methionine in rabbit reticulocyte lysate were performed according to the manufacturer's instructions (Promega, Madison, WI).

Degradation and Ubiquitination Assays—For *in vitro* protein degradation assays, reticulocyte lysate programmed to express recombinant proteins in the presence of [^{35}S]methionine (1 μl) was mixed with 9 μl of buffer or synchronized cell extracts. The reactions were supplemented with an energy regeneration system (25 mM phosphocreatine, 10 mg/ml creatine kinase, and 1 mM ATP). Unless stated otherwise, the reactions were incubated at 30 °C for 90 min and stopped with SDS-sample buffer and boiling. For *in vivo* ubiquitination assays, constructs expressing FLAG-tagged proteins were co-transfected with HA-Ub in pUHD-P2. The cells were treated with 50 μM of LLnL for 6 h before they were harvested. Cell extracts prepared from the transfected cells were immunoprecipitated with either normal rabbit serum or rabbit anti-FLAG polyclonal antibodies. The presence of HA-Ub-conjugated proteins in the immunoprecipitates was detected by immunoblotting with the anti-HA monoclonal antibody 12CA5.

Antibodies and Immunological Methods—Immunoblotting and immunoprecipitation were performed as described previously (33). Rat monoclonal antibodies YL1/2 against mammalian tubulin, monoclonal antibody HE12 against cyclin E1, monoclonal antibody E23 against cyclin A2 (31), monoclonal antibody A17 against CDC2 (34), anti-PSTAIRE monoclonal antibody (35), and rabbit anti-FLAG polyclonal antibodies (30) were obtained from sources as described previously. Monoclonal antibody GNS1 against cyclin B1 (sc-245), rabbit anti-cyclin F polyclonal antibodies (sc-952), goat anti-SKP1 antibodies (sc-1568), and goat anti-SKP2 antibodies (sc-1567) were from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal antibody M2 against FLAG tag was obtained from Sigma, and monoclonal antibody 12CA5 against HA tag was from Roche Molecular Biochemicals.

RESULTS

Cyclin F Is Destroyed at a Similar Time as Cyclin A but Is Not Affected by Replication and Spindle Checkpoints—We first confirmed that the antibodies that we used recognize endogenous cyclin F. Total HeLa cell extracts and cyclin F immuno-

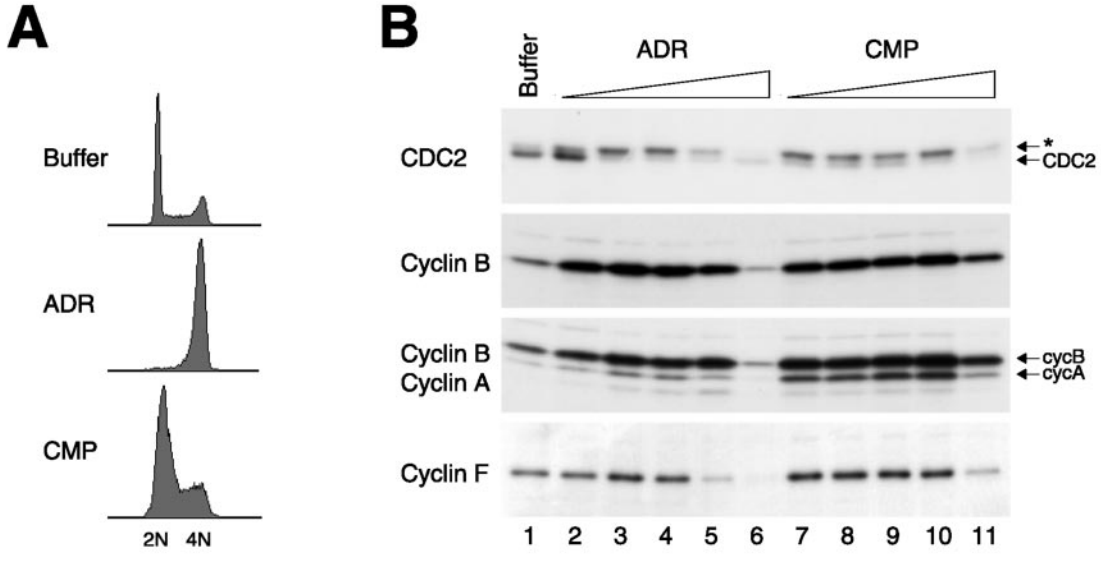

FIG. 3. **Cyclin F increases after DNA damage.** *A*, DNA damage-induced cell cycle arrest. Cells were treated with buffer, ADR (0.07 μg/ml), or CMP (0.7 μM) for 24 h. The cells were processed for flow cytometry analysis as described under "Experimental Procedures." *B*, increase of cyclin F after DNA damage. Cells were treated with buffer (*lane 1*) or increasing dosage of ADR (3-fold serial increase from 7 ng/ml in *lane 2* to 0.6 μg/ml in *lane 6*) and CMP (3-fold serial increase from 0.07 μM in *lane 7* to 6 μM in *lane 11*) for 24 h. Cell extracts were prepared and CDC2, cyclin B, cyclin A, and cyclin F were detected by immunoblotting. The *asterisk* indicates the slower migrating, phosphorylated form of CDC2.

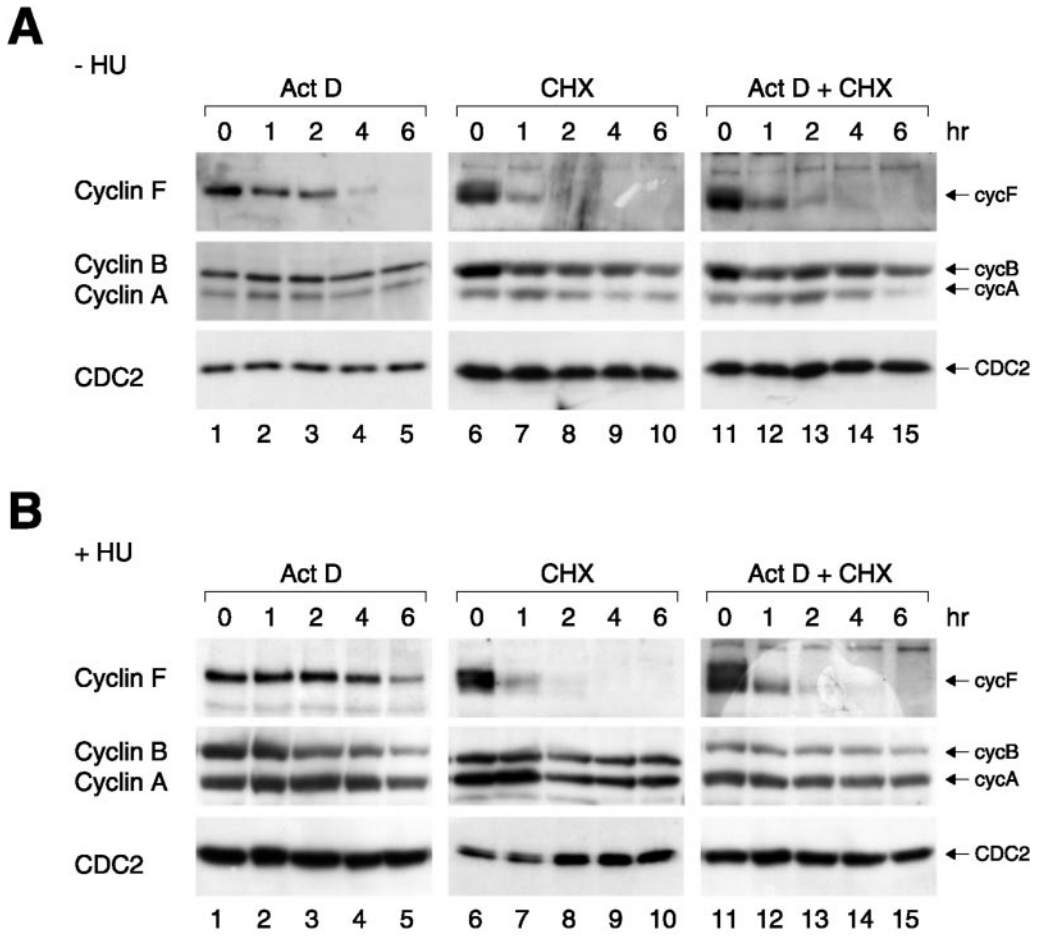

FIG. 4. **Endogenous cyclin F is unstable.** Asynchronous growing (*A*) or S phase-arrested (*B*) cells were treated with actinomycin D (*Act D*), cycloheximide (*CHX*), or both reagents together. Cell extracts were prepared at the indicated time points and were subjected to immunoblotting with antibodies against cyclin F, cyclin A, cyclin B, and CDC2.

precipitates were subjected to immunoblotting. Fig. 1*A* shows that ~100-kDa proteins were detected in total extracts and in cyclin F immunoprecipitates but not in immunoprecipitates using normal rabbit serum or serum against SKP2 (another F-box protein). The bands recognized by the antibodies comigrated with recombinant cyclin F (data not shown).

We next looked at the oscillation of cyclin F during G_2 phase in relation to other mitotic cyclins and to see whether cyclin F is affected by checkpoints. HeLa cells were first blocked in early S phase with a double thymidine block method and then synchronously released. We trapped the cells in the subsequent mitosis with nocodazole. As expected, cyclin A was destroyed during G_2 phase, and cyclin B was stabilized during the nocodazole-induced spindle assembly checkpoint (Fig. 1*B*). Flow cytometry indicated that these cells were synchronously released from the early S phase trap and reached G_2 phase by 6 h (Fig. 1*C*). In comparison with cyclin A, cyclin F was synthesized and destroyed more abruptly. Similar to cyclin A but unlike cyclin B, cyclin F was not stabilized by the spindle checkpoint. The relatively constant level of CDC2 served as gel loading control.

Given that both cyclin A and cyclin B accumulated when cells were blocked in S phase, we next examined whether cyclin F was affected by the DNA replication checkpoint. Fig. 2*A* shows that the levels of cyclin A, cyclin B, and cyclin E were higher in hydroxyurea-treated cells than in control cells. In contrast, similar level of cyclin F was present in these samples, indicating that cyclin F was not stabilized by the DNA replication checkpoint.

Cyclin F Is Degraded through Proteasome-independent Mechanisms—To see whether the degradation of cyclin F involves proteasomes, cells were treated with nocodazole in the presence or absence of the proteasome/calpain inhibitor LLnL. As expected, cyclin A, cyclin E, and cyclin F were destroyed after nocodazole treatment, but cyclin B was stabilized (Fig. 2*B*, *lane 2*). The destruction of cyclin A and cyclin E requires the proteasome and was abolished by the presence of LLnL (*lane 4*). In marked contrast, cyclin F was still destroyed in the presence of nocodazole and LLnL. The relatively constant level of tubulin acted as controls for sample loadings. For comparison, the levels of SKP1 (a protein that binds to the F-box region of cyclin F) and SKP2 were not affected by nocodazole and/or LLnL.

Accumulation of Cyclin F during G_2 and S DNA Damage Checkpoints—Adriamycin (ADR) and camptothecin (CMP) are inhibitors of topoisomerase II and topoisomerase I, respectively. Both reagents eventually induce double-stranded breaks in the DNA, but the action of CMP appears to be limited to S phase (reviewed in Ref. 36). ADR-arrested HeLa cells in G_2 phase and CMP-arrested cells predominantly in S phase (Fig. 3*A*). CDC2 shifted to a slower migrating form that represented Thr^{14}/Tyr^{15} phosphorylation and inactivation of its kinase activity (Fig. 3*B*). Furthermore, both cyclin A and cyclin B accumulated after ADR and CMP treatments. The levels of cyclin A and cyclin B declined at higher dosage of ADR and CMP, probably due to cell death at these levels of DNA damage. Interestingly, cyclin F also accumulated after ADR or CMP treatments, mirroring closely the inactivation of CDC2.

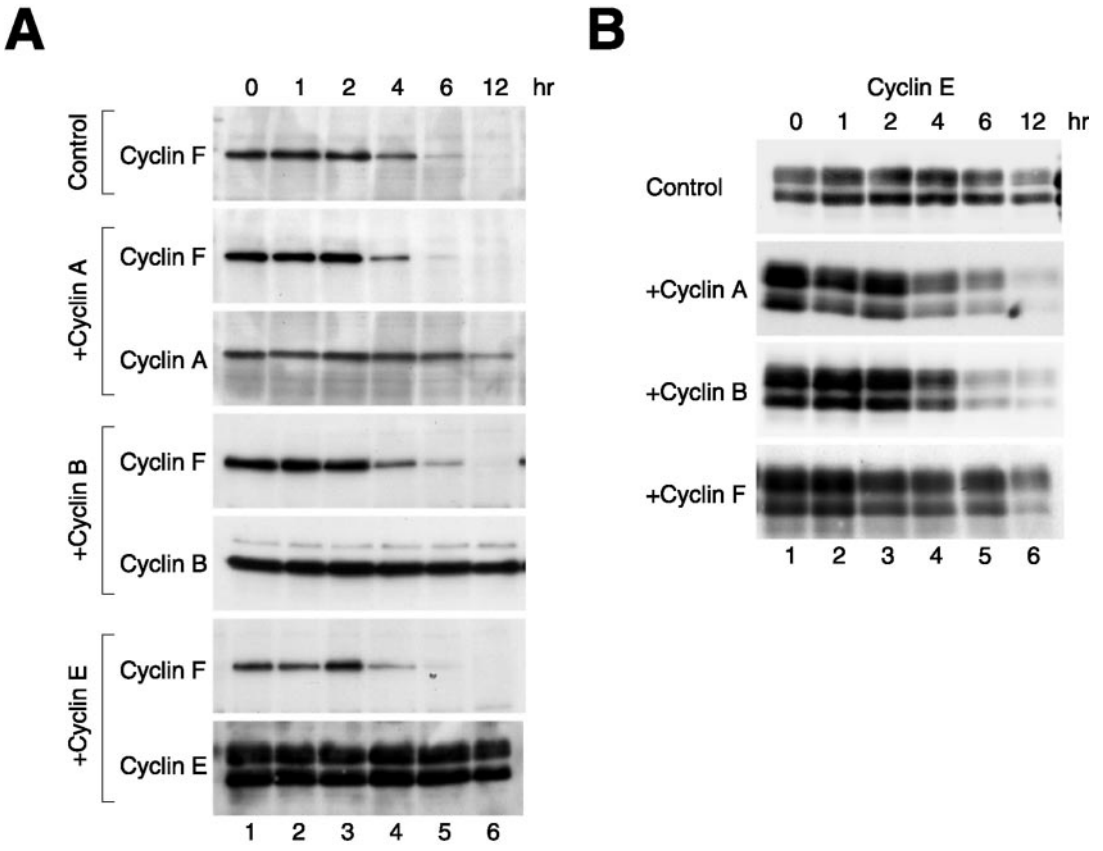

FIG. 5. **The stability of cyclin F is not affected by other cyclins.** *A*, the stability of cyclin F is not affected by co-transfection with other cyclins. Control vector or plasmids expressing cyclin A, cyclin B, or cyclin E were co-transfected with FLAG-cyclin F in pUHD-P1 as indicated. At 24 h after transfection, doxycycline (*Dox*) was added to turn off the promoter expressing FLAG-cyclin F. At the indicated time points after the addition of Dox, cell extracts were prepared and immunoblotted with antibodies against FLAG (for cyclin F) and other cyclins. *B*, the stability of cyclin E is reduced in the presence of cyclin A and cyclin B but not by cyclin F. Control vector or plasmids expressing cyclin A, cyclin B, or cyclin F were co-transfected with cyclin E in pLINX as indicated. At 24 h after transfection, doxycycline was added to turn off the promoter expressing cyclin E. At the indicated time points after the addition of Dox, cell extracts were prepared, and cyclin E was detected by immunoblotting.

Taken together, these data showed that the important checkpoints that affect cells from S phase to mitosis have different effects on different cyclins. The A-, B-, and E-type cyclins are stabilized by the DNA replication checkpoint. In contrast, only cyclin B is stabilized by the spindle assembly checkpoint. Finally, the A-, B-, and F-type cyclins accumulated during the DNA damage checkpoints that delay S phase and G_2 phase.

Cyclin F Is an Unstable Protein in Vivo—We next analyzed the stability of the endogenous cyclin F in relation to other cyclins. When *de novo* protein synthesis was inhibited with cycloheximide, cyclin F disappeared in less than 1 h (Fig. 4*A*, *lanes 6–10*), suggesting that cyclin F was a very unstable protein in growing cells. Cyclin F also decreased when transcription was blocked with actinomycin D, but this occurred more slowly than with cycloheximide (*lanes 1–5*). This indicates that cyclin F protein has a shorter half-life than its mRNA. Not surprisingly, adding actinomycin D and cycloheximide together induced the disappearance of cyclin F at similar rate as with cycloheximide alone. In contrast, cyclin A and cyclin B were more stable than cyclin F, and reduction of the proteins were only appreciable at later time points. The relatively stable protein CDC2 acted as a control for sample loading.

Since cyclin F starts to accumulate during S phase, we next investigated the stability of cyclin F in the presence of hydroxyurea (Fig. 4*B*). A similar short half-life of cyclin F protein was observed in S phase as in growing cells. However, the mRNA of cyclin F appeared to be more stable during S phase, since cyclin F remained elevated following actinomycin D treatment. We have not been able to examine the stability of cyclin F in G_2 phase due to the lack of a good nontoxic G_2 cell cycle blocker. Hence, given the short half-life of the protein, the major determinants of cyclin F level are probably due to transcription control and mRNA stability.

The Stability of Cyclin F Is Not Affected by Other Cyclins—It is hypothesized that the stability of one cyclin may be affected by the expression of another type of cyclin. This could be attributed to either a direct effect of the cyclin on the degradation machinery, or indirectly due to the cell cycle block imposed by the cyclin. We investigated the stability of cyclin F by putting its cDNA under the control of a doxycycline-responding promoter. The addition of doxycycline reduced the level of FLAG-tagged cyclin F to an undetectable level (Fig. 5*A*). We found that co-transfection of FLAG-cyclin F with constitutive expression plasmids for cyclin A, cyclin B, or cyclin E (also shown by immunoblotting) did not affect the stability of cyclin F. For comparison, similar experiments performed with cyclin E showed that its stability was reduced when co-expressed with cyclin A or cyclin B but not with cyclin F (Fig. 5*B*). One implication from these results is that the stability of cyclin F is not sensitive to cell cycle arrest caused by overexpression of other cyclins.

Rapid Degradation of Cyclin F Requires the COOH-terminal PEST Sequence but Not the N-terminal F-box—Cyclin F contains two structural elements that are generally connected to proteolysis: an F-box motif at the N terminus and the PEST sequences at the COOH-terminal quarter of the protein. We used an *in vitro* degradation system to elucidate the relationship between these structural elements and cyclin F stability.

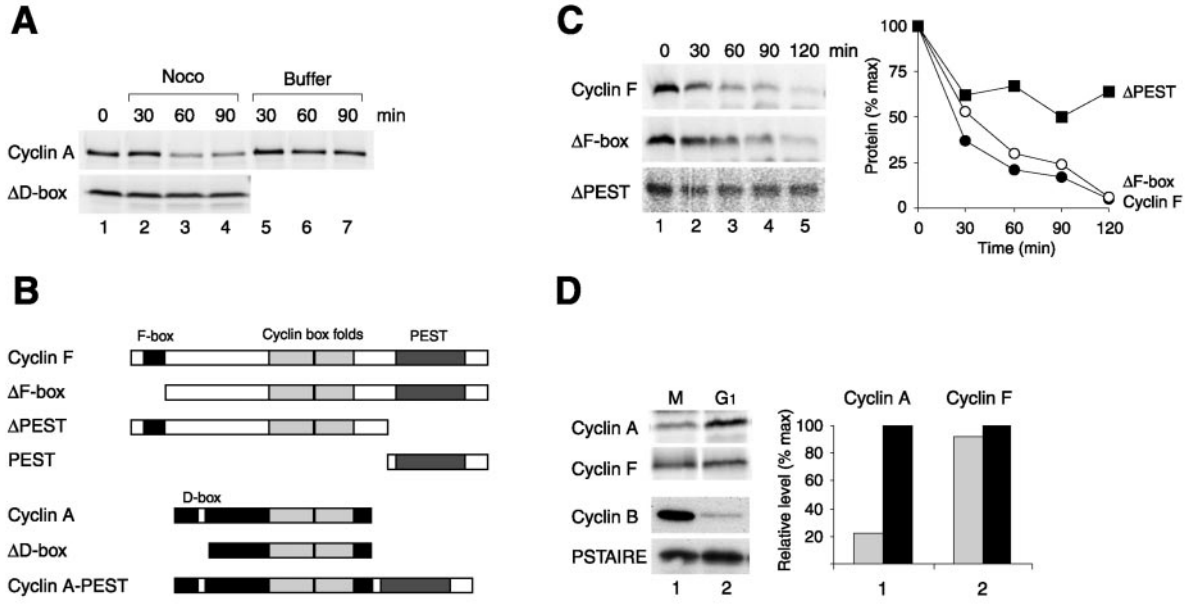

FIG. 6. ***In vitro* degradation of cyclin A and cyclin F.** *A*, *in vitro* cyclin degradation assays. *In vitro* translated cyclin A or cyclin A(NΔ71) was incubated with nocodazole-blocked HeLa cell extracts as described under "Experimental Procedures." Cyclin A was also incubated with buffer as controls (*lanes 5–7*). At the indicated time points, samples were taken for SDS-PAGE, and radiolabeled proteins were detected with phosphorimaging. *B*, schematic diagram of the cyclin F and cyclin A constructs used in this study. The positions of the various structural elements are shown to scale. *C*, rapid degradation of cyclin F *in vitro* depends on the PEST but not the F-box region. *In vitro* degradation assays were preformed with radiolabeled full-length cyclin F, F-box deletion mutant (ΔF-box), or PEST region deletion mutant (ΔPEST) in nocodazole-blocked HeLa cell extracts as described under "Experimental Procedures." The signals were quantified with a PhosphorImager and plotted on the *right*. *D*, cyclin F is degraded with similar efficiency in mitotic and G_1 cell extracts. Cyclin A and cyclin F produced in rabbit reticulocyte lysates were mixed with extracts prepared from cells in mitosis (*lane 1*) or G_1 phase (*lane 2*). The relative amount of cyclin A and cyclin F after 60 min of incubation was detected by SDS-PAGE and phosphorimaging. Quantifications of cyclin A and cyclin F are shown on the *right* (*gray*, mitosis; *black*, G_1). The endogenous cyclin B and CDC2/CDK2 were detected by immunoblotting.

Radiolabeled cyclin A or cyclin F was produced in a coupled transcription-translation reticulocyte lysate system. Recombinant cyclin A was destroyed when it was mixed with extracts from nocodazole-blocked cells (Fig. 6*A*). Deletion of the N-terminal region of cyclin A that contains the destruction box sequence (ΔD-box) (see Fig. 6*B*) abolished its degradation, suggesting that the degradation was not due to nonspecific proteolysis.

Fig. 6*C* shows that cyclin F was also degraded using the same *in vitro* system. Moreover, degradation of cyclin F was not affected by removing the F-box region (ΔF-box). Importantly, the stability of cyclin F was substantially increased when the PEST-containing COOH-terminal region was removed (ΔPEST).

We found that whereas cyclin A was degraded by mitotic extracts, it was relatively stable in G_1 cell extracts (Fig. 6*D*). In contrast, cyclin F was similarly degraded in both mitotic and G_1 cell extracts. Immunoblotting of the extracts for cyclin B and PSTAIRE confirmed that the G_1 cells had exited mitosis and that similar amounts of extracts were used, respectively. These data indicate that whereas the destruction machinery for cyclin A was active during mitosis but not in G_1, the mechanism that degraded cyclin F was equally active during mitosis and G_1, further testifying to the difference between the degradation of cyclin A and cyclin F.

To confirm that deletion of the F-box region did indeed disrupt the interaction between cyclin F and SKP1, we looked at whether recombinant cyclin F could bind to SKP1. Fig. 7*A* shows that endogenous SKP1 was co-immunoprecipitated with FLAG-cyclin F but not with immunoprecipitates of control normal rabbit serum. For comparison, SKP1 was also co-immunoprecipitated with SKP2. It is known that SKP2 can form a complex with cyclin A-CDK complexes (37), as was confirmed in Fig. 7*A*. Interestingly, no interaction between cyclin F and cyclin A-CDK complexes was detected under similar conditions. Significantly, cyclin F lacking the N terminus (ΔF-box) did not form a complex with SKP1 (Fig. 7*B*).

Cyclin F Is Not Ubiquitinated in Vivo—We used an *in vivo* ubiquitination assay to see whether cyclin F was ubiquitinated. FLAG-tagged cyclins were co-expressed with a HA-tagged ubiquitin (HA-Ub), and proteasome inhibitors were added to stabilize the ubiquitinated proteins. A smear of high molecular weight, HA-containing proteins in the FLAG immunoprecipitates indicated conjugation of HA-Ub to the cyclins (Fig. 8). As expected, both cyclin A and cyclin B were ubiquitinated *in vivo*. In contrast, no HA-Ub-conjugated protein was seen with FLAG-cyclin F. As controls, neither FLAG-tagged cyclins nor HA-Ub-conjugated proteins was immunoprecipitated with normal rabbit serum. Based on this and other evidence shown above, we interpret that in contrast to other cyclins, cyclin F was not targeted by the ubiquitin/proteasome system.

The PEST Sequence of Cyclin F Is Sufficient to Confer Instability When Transferred to Cyclin A—Given that cyclin F was not degraded through the ubiquitin/proteasome pathway, what are the proteases that are responsible for the rapid turnover of cyclin F? To begin to address this question, inhibitors of various classes of proteases were added to the *in vitro* degradation assay for cyclin F. Fig. 9*A* shows that the only reagents that significantly inhibited the degradation of cyclin F were EDTA and EGTA.

The above datum suggests that degradation of cyclin F may involve proteases that use metal ions as active centers (metalloproteases) or other metal-dependent proteases like calpain. It is known that proteolysis of several PEST-containing proteins like IκB and γ_c involves calpain (16, 17). Although degradation of cyclin F was inhibited by EGTA, we do not favor the idea that calpain is responsible for the rapid turnover of cyclin F. This is because the cysteine protease inhibitor E64 did not inhibit the

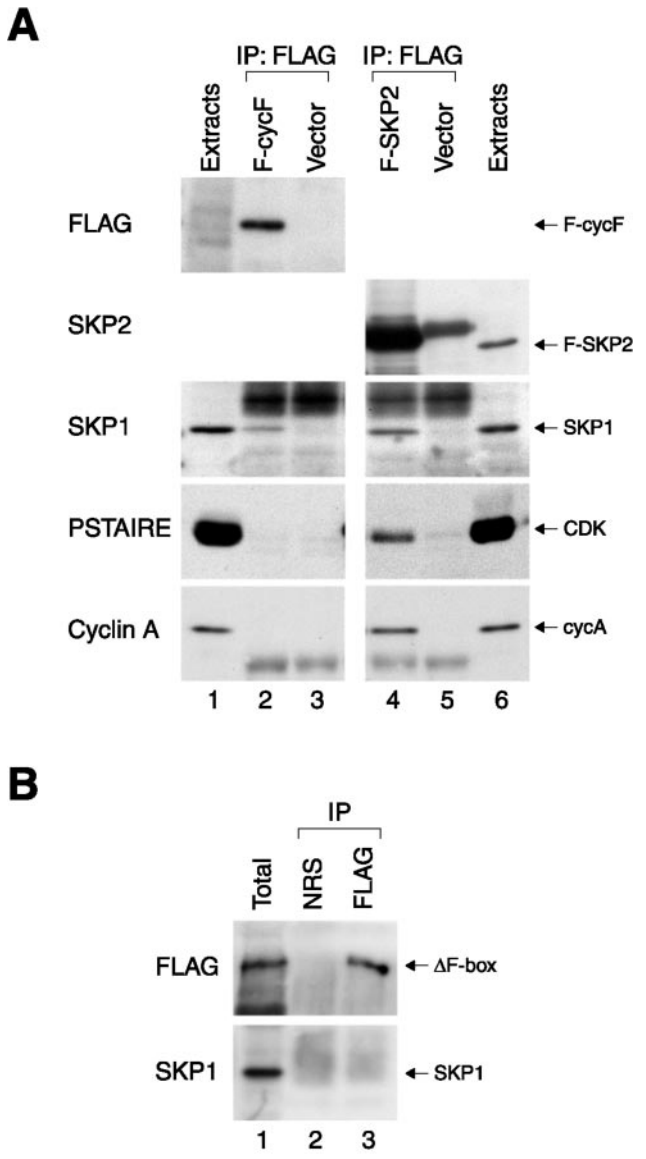

FIG. 7. **Cyclin F lacking the F-box region cannot bind SKP1.** *A*, cells were transfected with plasmids expressing FLAG-cyclin F (*lane 2*), FLAG-SKP2 (*lane 4*), or control vectors (*lanes 3* and *5*). Cell extracts were then prepared, and 200 μg were immunoprecipitated with anti-FLAG tag serum. Samples were applied onto SDS-PAGE and immunoblotted with antibodies against FLAG, SKP2, SKP1, PSTAIRE (which detected both CDC2 and CDK2), and cyclin A as indicated. Total HeLa cell extracts (10 μg) were loaded in *lanes 1* and *6*. *B*, cells were transfected with plasmids expressing FLAG-cyclin F(ΔF-box). Cell extracts were prepared, and 200 μg were immunoprecipitated with normal rabbit serum (*lane 2*) or anti-FLAG serum (*lane 3*). Samples were applied onto SDS-PAGE and immunoblotted with antibodies against FLAG and SKP1.

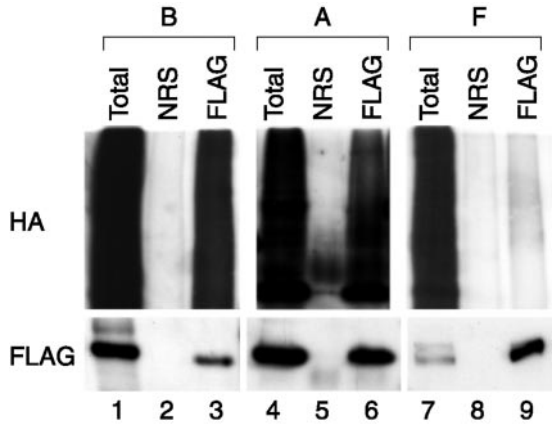

FIG. 8. **Cyclin F is not ubiquitinated *in vivo*.** HA-tagged Ub was co-expressed with FLAG-tagged cyclin B (*lanes 1–3*), cyclin A (*lanes 4–6*), or cyclin F (*lanes 7–9*). Cell extracts were prepared and immunoprecipitated with either normal rabbit serum (*lanes 2*, *5*, and *8*) or anti-FLAG serum (*lanes 3*, *6*, and *9*). Total cell extracts were loaded in *lanes 1*, *4*, and *7*. The samples were subjected to immunoblotting with antibodies against HA (*upper panel*) and FLAG (*lower panel*).

degradation of cyclin F (Fig. 9*A*). Moreover, the addition of the specific calpain inhibitor LLM did not stabilize cyclin F *in vivo* (Fig. 9*B*). The addition of the calpain/proteasome inhibitor LLnL slightly stabilized cyclin F (Figs. 2*B* and 9*B*), but LLnL clearly could not inhibit the degradation of cyclin F in the presence of nocodazole (Fig. 2*B*). Taken together, it is possible that degradation of cyclin F may involve metalloproteases.

To see whether the PEST region of cyclin F was sufficient to act as an independent unit that influences protein stability, we subcloned the PEST region alone into a mammalian expression plasmid and investigated whether it could affect the turnover of cyclin F. When FLAG-tagged PEST was co-expressed with cyclin F (with FLAG-PEST in excess), the stability of cyclin F was higher than when it was expressed alone (Fig. 9*C*). This suggests that the excess PEST could interfere with the degradation of full-length cyclin F, possibly through competition with the endogenous proteases. One caveat to this experiment is that the half-life of recombinant cyclin F was higher than that of the endogenous cyclin F. This is probably because cyclin F produced in these transient transfections was substantially more abundant than the endogenous protein; hence, expression of cyclin F itself may already be straining the proteolytic system.

We next investigated whether the PEST region could confer instability when transferred to another protein. Cyclin A, which was relatively stable outside M phase (Fig. 4), was fused to the PEST region of cyclin F (cyclin A-PEST). We found that the fusion protein was substantially less stable than cyclin A (Fig. 9*D*). Taken together, these data show that the PEST region of cyclin F is sufficient to decrease the stability of heterogeneous protein.

DISCUSSION

Cell Cycle Control of Cyclin F—Among the three cyclins that are synthesized and destroyed during G_2 and mitosis (cyclin A, cyclin B, and cyclin F), the function and control of cyclin F is the most elusive. Cyclin B-CDC2 is the classic M phase-promoting factor that drives entry into mitosis. Cyclin A can activate both CDC2 and CDK2 and functions in S phase and mitosis (38). Despite the fact that the cyclin box of cyclin F is most similar to cyclin A, no CDK partner has been identified for cyclin F. The one function proposed for cyclin F is its ability to bind cyclin B and transport it into the nucleus (5).

The synthesis and destruction of cyclin F are typically more abrupt than for cyclin A and cyclin B. We observed that cyclin F was consistently destroyed slightly later than cyclin A in HeLa cells. This was observed when cells were synchronously released from S phase into a mitotic block (Fig. 1) or when cells were allowed to progress through mitosis (data not shown). The tight temporal regulation of cyclin F points to a potentially important role in the cell cycle.

Both synthesis and destruction of cyclins are important for cell cycle progression. It is well known that destruction of cyclin B by anaphase-promoting complex/cyclosome is essential for metaphase-anaphase transition, and expression of a indestructible cyclin B traps cells in mitosis. Similarly, expression of indestructible cyclin A arrests cells in late mitosis (14, 39). Overexpression of cyclin F (or mutant lacking PEST) also

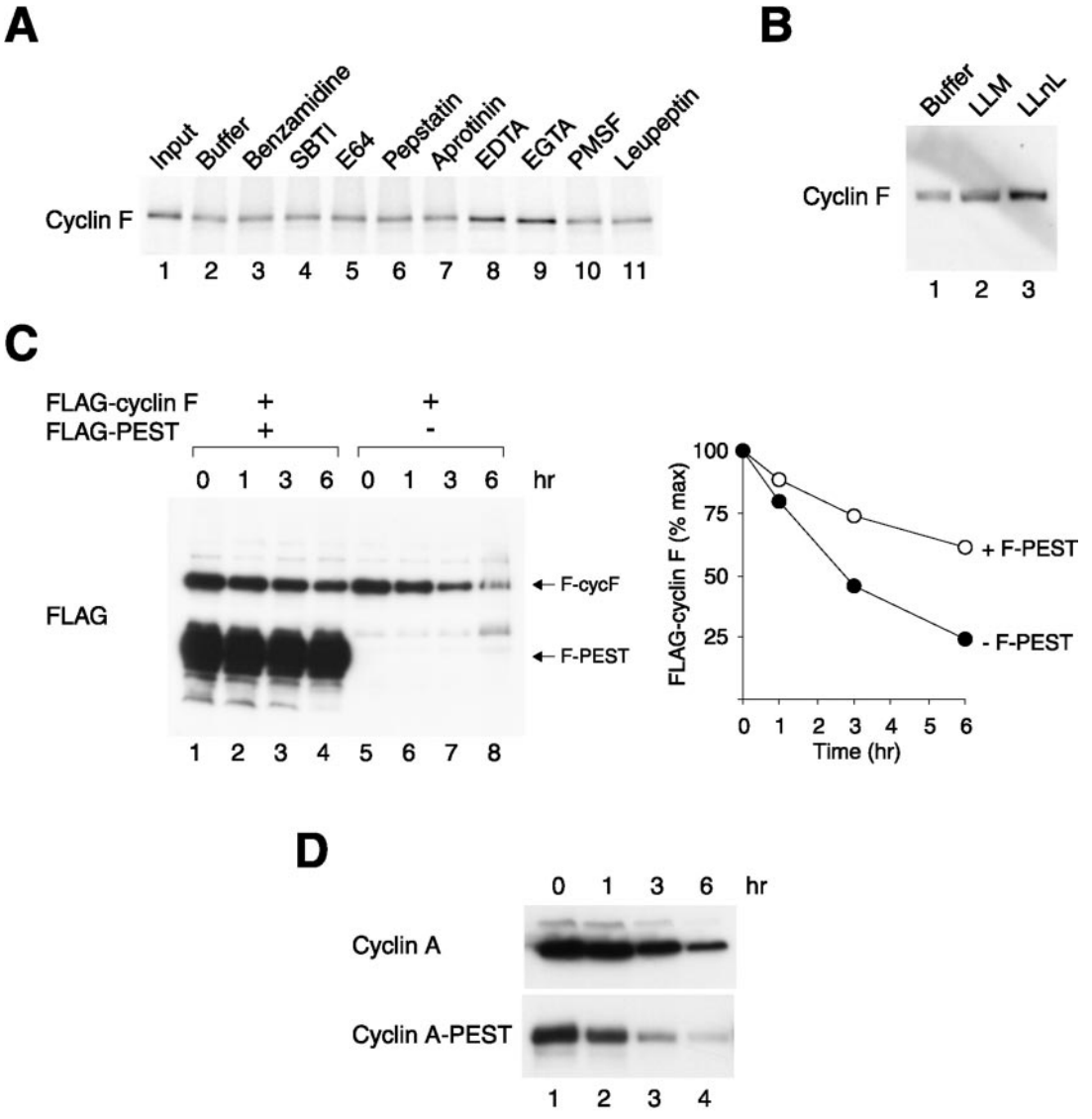

FIG. 9. **Characterization of the PEST sequences of cyclin F.** *A*, degradation of cyclin F *in vitro* is inhibited by EDTA and EGTA. *In vitro* translated cyclin F was mixed with nocodazole-blocked cell extracts as described under "Experimental Procedures." The reactions were supplemented with control buffer, benzamidine (100 μg/ml), SBTI (50 μg/ml), E64 (10 μM), pepstatin (1 μM), aprotinin (2 μg/ml), EDTA (10 mM), EGTA (10 mM), phenylmethylsulfonyl fluoride (1 mM), leupeptin (100 μM) as indicated. The input and the cyclin F remained after the incubation were analyzed by SDS-PAGE and phosphorimaging. *B*, buffer, LLM, or LLnL was added to growing HeLa cells as indicated. Cell extracts were prepared after 6 h, and the relative level of cyclin F was detected by immunoblotting. *C*, FLAG-cyclin F was co-transfected with FLAG-PEST (*lanes 1–4*) or control vector (*lanes 5–8*). Cycloheximide was added to inhibit protein synthesis at 24 h after transfection. At the indicated time points after cycloheximide addition, cell extracts were prepared and the FLAG-tagged proteins were detected by immunoblotting. The signals were quantified with the NIH Image program and plotted on the *right*. *D*, cells were transfected with plasmids expressing FLAG-cyclin A or FLAG-cyclin A-PEST. Cycloheximide was added to inhibit protein synthesis at 24 h after transfection. At the indicated time points after cycloheximide addition, cell extracts were prepared, and FLAG-tagged proteins were detected by immunoblotting.

causes an accumulation of the G_2/M population (6), but it is not known whether the cells are delayed at any particular point during mitosis. Why is destruction of cyclin F important for G_2/M? Since the nuclear envelope is broken down following the activation of cyclin B-CDC2 complexes, cyclin F is no longer required for cyclin B transport after its activation. This suggests that cyclin F has functions in addition to transporting cyclin B into the nucleus. Another possibility is that the presence of cyclin F-cyclin B complexes during mitosis could interfere with the normal functions of cyclin B.

Activation of the spindle assembly checkpoint, which delays metaphase-anaphase transition until all chromosomes are attached to the mitotic spindles, inhibits cyclin B but not cyclin A degradation (14, 39). We found that, similar to cyclin A, cyclin F was not stabilized by the spindle assembly checkpoint. Ectopic expression of cyclin F did not affect the ability of the cell to activate the spindle assembly checkpoint.[2]

As with cyclin A, cyclin F also accumulated slightly after DNA damage. This was not merely due to an increase of cells in G_2 phase, since camptothecin mainly arrested cells in S phase. We have not directly determined the stability of cyclin F following DNA damage because of the substantial cell death induced by the DNA-damaging agents and cycloheximide together. In the case of cyclin A, increase in the protein appears to be important for DNA damage checkpoints in some cells. *Drosophila* cells lacking cyclin A are unable to delay in mitosis after DNA damage, and they enter anaphase with an increased

[2] T. K. Fung, W. Y. Siu, C. H. Yam, A. Lau, and R. Y. C. Poon, unpublished data.

number of lagging chromosomes (40). The potential role of cyclin F after DNA damage awaits further investigation.

Degradation of Cyclins—Degradation of the mitotic cyclins requires a D-box, which acts as a signal for ubiquitin/proteasome-mediated proteolysis (9, 10). Despite the similarity in timing between the destruction cyclin F and the mitotic cyclins, no obvious D-box is present in cyclin F. Cyclin D and cyclin E lack the D-box but are also known to be degraded through the ubiquitin/proteasome-mediated pathway (21, 22, 41). We demonstrated that cyclin F was not ubiquitinated *in vivo*, and its destruction was not inhibited by proteasome inhibitors. Furthermore, we have no evidence that cyclin F is ubiquitinated using an *in vitro* ubiquitination assay.[2]

In vivo and *in vitro* degradation assays suggest that cyclin F has a short half-life throughout much of the cell cycle. Unfortunately, it is technically difficult to address the important question of whether the stability of cyclin F changes during G_2 phase (when cyclin F normally accumulates) due to the lack of a good nontoxic G_2 cell cycle blocker. *In vitro* degradation assays using synchronized cell extracts suggest that there is no significant change in the stability of cyclin F from S phase to the subsequent G_1 phase.[2]

Degradation of F-box Proteins—Cyclin F can associate with SKP1 (Fig. 7) as well as CUL1 and RBX1[2] to form a SCF complex. It is generally believed that proteins containing F-box are part of the SCF ubiquitin ligase complex that targets different proteins for ubiquitination (20). At this stage, it is unclear what proteins are ubiquitinated by $SCF^{cyclin\ F}$ complexes.

Similar to cyclin F, many F-box proteins oscillates during the cell cycle. For example, the SKP2 level is high in S phase and low in M phase (37). Variation of the F-box protein may be an important way to control the activity of the ubiquitin ligase for a particular substrate. Whereas it may not be the rule, several F-box proteins like SKP2 (28), Grr1p, and Cdc4p (26) are known to be degraded via ubiquitination. Although cyclin F appears to use a completely different mechanism for degradation, the down-regulation of cyclin F is nevertheless very abrupt and efficient, in part due to the short half-life of the protein.

Degradation of PEST-containing Proteins—The presence of the PEST sequences in cyclin F has been proposed to destabilize the protein (6). Here we show that cyclin F was indeed more stable when the PEST region was removed. Moreover, overexpression of the PEST region alone competed for the degradation of cyclin F, and fusion of the PEST region to cyclin A rendered the protein less stable. These data suggest that the PEST region is both necessary and sufficient to destabilize cyclin F.

Many unstable proteins contain PEST sequences (4), but the molecular basis of their instability is a mystery. It is unlikely that a single common mechanism is involved in their degradation. Interestingly, degradation of cyclin D1 after retinoic acid treatment of epithelial cells is dependent on the ubiquitin/proteasome pathway and the PEST region (42). Deletion of the PEST sequence stabilizes cyclin D1, but it is not clear whether this affects the ubiquitination. In contrast, the PEST sequences of the retinoid X receptor α are not required for its ubiquitin/proteasome-mediated proteolysis (43). Proteasome is not necessarily involved in the degradation of PEST-containing proteins, as for cyclin T1 (44) and cyclin F described here. Proteolysis of many PEST-containing proteins involves calpain (16, 17). For IκBα, two mechanisms of degradation have been described, one through the ubiquitin/proteasome pathway and another through calpain. The binding of the PEST sequence of IκBα to the calmodulin-like domain μ-calpain is critical for its calpain-dependent degradation (16). We believe that the degradation of cyclin F is independent of calpain (Fig. 9), which is also the case for some other PEST-containing proteins (18, 19).

The PEST sequences of many proteins are phosphorylated. As exemplified by IκBα, degradation of IκBα is dependent on phosphorylation of its PEST sequence by IκK (45, 46). Similarly, phosphorylation of Thr^{286} in the PEST sequence of cyclin D1 is also required for its ubiquitin/proteasome-mediated proteolysis (41). Cyclin F typically appeared as multiple bands (depending on the quality of the gels), suggesting that it was heavily phosphorylated. These mobility shifts were more readily observable with the cyclin A-PEST fusion and the FLAG-PEST. This is not too surprising, since PEST sequences contain a large number of serine and threonine residues. Determination of whether these phosphorylations contribute to the degradation of cyclin F will require further investigation.

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