Ubiquitination of full-length cyclin

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Abstract Mitotic cyclins are key cell-cycle regulators that are relatively stable through most of the cell-cycle then rapidly degraded at mitosis. We have detected ubiquitin conjugates of full-length *Xenopus* cyclin B2 strongly suggesting that ubiquitination rather than a proteolytic cleavage is the initiating event in cyclin destruction. The highest levels of ubiquitin conjugates correlate with the phase of rapid proteolysis. This result supports previous findings that implicate the ubiquitin system in cyclin proteolysis. However, we also observe cyclin-ubiquitin conjugates in both cytostatic factor arrested and interphase extracts where cyclin is more stable. The physiologic role of ubiquitinated cyclin under these conditions is unclear.

Key words: Ubiquitin; Cyclin B2

1. Introduction

Cyclins function as subunits of kinases essential for normal cell-cycle regulation [1]. Cyclin B2 is a mitotic cyclin that binds the $p34^{cdc2}$ kinase to regulate M-phase promoting factor (MPF) activity [2]. Although relatively stable throughout most of the cell-cycle, mitotic cyclins are abruptly degraded during mitosis, and this proteolytic event is critical for completion of mitosis [2,3]. We have used *Xenopus* egg extracts to study the mechanisms underlying cyclins' rapid destruction. *Xenopus* eggs are naturally arrested in metaphase of meiosis II, in which cyclin is stable and MPF activity is constituitively high. The agent responsible for this metaphase arrest is termed cytostatic factor (CSF) and accordingly this physiologic state is referred to as CSF arrest. Fertilization results in cytosolic Ca^{2+} fluxes that override the CSF arrest and initiate a host of events related to the resumption of cell-cycle progression: MPF activity drops, anaphase commences, and mitotic cyclins are degraded [4]. Extract prepared from unactivated eggs exhibits the CSF arrest and can be experimentally manipulated by Ca^{2+} addition to mimic all of the events associated with release from CSF arrest [3].

The ubiquitin (Ub) dependent proteolytic system is involved in degrading several cytoplasmic proteins [5]. Ub can be covalently coupled via its carboxy terminus to the epsilon amino groups of lysine residues in cellular proteins, thereby targeting them for destruction. The Ub system consists of an elaborate array of enzymes: a series of Ub conjugating enzymes (E1, E2s and E3s); a family of isopeptidases that can remove Ub from conjugated proteins; and a 26S protease that degrades ubiquitinated proteins [5,6]. An important property of Ub is its ability to conjugate to itself thereby forming extended chains on the target protein; substrates conjugated to long Ub chains are more efficiently degraded [7]. This selection is due to the Ub-conjugate binding properties of a 26S protease subunit that exhibits a higher affinity for polymers containing four or more Ub molecules [8].

The need for Ub polymers to induce substrate proteolysis has been exploited by Hershko and Heller to inhibit Ub dependent degradation [9]. They reductively methylated Ub to block all lysine epsilon amino groups. The resulting methylated Ub (MeUb) can be conjugated to target proteins, but cannot support chain elongation. For some substrates, proteolysis is severely inhibited.

Several lines of evidence implicate the Ub system in cyclin proteolysis. Glotzer et al. working with *Xenopus* egg extract, demonstrated that a cyclin-protein A fusion protein is ubiquitinated more in mitotic extract than during interphase [10]. Hershko et al. showed that MeUb inhibited cyclin proteolysis in clam extract; however, they did not detect cyclin-MeUb conjugates [11]. More recently Sudakin et al. have fractionated the clam extract and identified the cyclosome: a 1500 kDa complex that is cell-cycle regulated and was partially purified based on its ability to ubiquitinate cyclin fragments [12]. They also demonstrated that the cyclosome could ubiquitinate bacterially expressed full-length cyclin A purified from inclusion bodies. Using *Xenopus* extracts, King et al. [13] confirm the existence of a 20S complex that, like the clam cyclosome, is activated at mitosis and required for cyclin ubiquitination.

In these studies, we have detected Ub conjugates of full-length *Xenopus* cyclin B2, thus indicating that prior proteolytic cleavage of cyclin is not necessary for its ubiquitination. The concentration of Ub-cyclin adducts is highest during the phase of rapid cyclin degradation, but Ub-cyclin conjugates can also be detected during CSF arrest and interphase.

2. Materials and Methods

Creatine phosphokinase, ATP, phosphocreatine, $CaCl_2$, calf liver tRNA, EGTA, cycloheximide and cytochalasin B were purchased from Sigma. Versilube oil was from Andpak-EMA. Amino acid mix minus methionine, T7 RNA polymerase, DTT, rNTPs and RNasin were from Promega. RNase A, RNase inhibitor, 10 × transcription buffer and *Sph*I were from Boehringer Mannheim. $m^7G(5')$ ppp(5')G was from New England Biolabs. [^{35}S]Methionine was from DuPont-New England Nuclear. Rainbow markers were from Amersham.

2.1. Preparing CSF arrested extract

Xenopus laevis frogs were induced to ovulate as described previously [14]. CSF arrested extract was prepared as described previously [3]. Briefly, dejellied eggs were washed 4 times with 100 mM KCl, 0.1 mM $CaCl_2$, 1.0 mM $MgCl_2$, 50 mM sucrose, 10 mM HEPES pH 7.7 and twice with the same buffer containing 5 mM EGTA and 0.1 mg/ml cytochalasin B. The eggs were spun through versilube oil, for 1 min at 1000 rpm and 30 s at 2000 rpm, then crushed by centrifugation at 10,000 rpm for 10 min in a Beckman SW50.1 rotor at 16°C. Extract was collected by side puncture, supplemented with cytochalosin B to 10 μg/ml and an ATP regenerating system consisting of 10.1 mM ATP,

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288 units/ml creatine phosphokinase, 132 mM phosphocreatine, 200 mM Tris-HCl, 10mM $MgCl_2$, 20 mM KCl, 1 mM DTT, pH 7.8 was added (1/20, v/v). The extract was microfuged for 10 min at 4°C, then held on ice for 1–2 h before use.

2.2. Preparation of cyclin B2 mRNA

A plasmid containing a full-length *Xenopus* cyclin B2 clone was generously provided by Tim Hunt [15]. The plasmid was linearized with *Sph*I. Ten μg of plasmid was added to a 100 μl transcription mix containing: 10 μl transcription buffer, 0.1 mM rGTP, 1 mM rATP, rCTP and rUTP, 5 units $m^7G(5')$ ppp(5')G, 12.5 mM DTT, 40 units RNasin, 40 units T7 polymerase. After 30 min at 37°C rGTP was added to a final concentration of 1.1 mM and the reaction was continued for another hour. The mRNA was ethanol precipitated, resuspended in water at 2–10 mg/ml and stored at −80°C.

2.3. Preparation of [^{35}S]methionine-labeled cyclin B2

Either interphase extract from electrically activated eggs (prepared as described previously [14]) or CSF arrested extract were used for producing in vitro translated cyclin. Both produce cyclin which is degraded in a cell-cycle dependent manner: CSF produced cyclin was used in Fig. 1; interphase produced cyclin in Figs. 2 and 3. Extract was prepared for in vitro translation by modifications of published procedures [16,17]. Extract was treated with RNase A at 0.25 μg/ml for 20 min at 10°C. Then DTT was added to 3 mM and RNasin (Promega) and RNase inhibitor (Boehringer Mannheim) were each added to a final concentration of 0.5 units/μl. Extract was incubated at 10°C for 10 min. Then tRNA was added to a final concentration of 0.1 mg/ml and an amino acid mixture minus methionine was added to 0.02 mM. [^{35}S]Methionine was added to a final concentration of 4.3 μCi/μl and the reaction was shifted to 23°C. Cyclin mRNA was added (final concentrations 0.25–0.6 mg/ml) and the reactions were allowed to proceed for either 1 h (CSF extract) or 40 min (interphase extract). Cyclin containing extract was stored at −80°C until use.

2.4. Methylated HisUb preparation

Six histidine residues were cloned in after the initial methionine of the Ub gene and the construct was placed under isopropyl-β-D-thiogalactopyranoside control using standard recombinant DNA procedures [18]. The resulting histidine tagged Ub (HisUb) was expressed in *Escherichia coli* and purified as described [19] except the heat treated supernatant was concentrated on a centricon 3 microconcentrator (Amicon) and purified on a superdex 75 16/60 sizing column (Pharmacia). Purified HisUb was methylated as described previously [9].

2.5. SDS-PAGE

Samples were dissolved in SDS sample buffer consisting of 0.0625 M Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, and 0.05% Bromophenol blue. Samples were run on 12.5% gels as described [20].

3. Results and discussion

Previous reports have implicated the Ub system in cyclin proteolysis [10–13]. Initial evidence, published by Glotzer et al. [10], demonstrated Ub conjugates of a cyclin-protein A fusion protein. Contemporaneous studies revealed that MeUb inhibited the degradation of clam cyclin, yet cyclin-Ub conjugates were not detected [11]. Additional experiments in which N-terminal cyclin fragments were used as proteolytic substrates yielded equivocal results with respect to Ub conjugates: Holloway et al. demonstrated Ub conjugates of the fragments [21], while van der Velden and Lohka did not detect them [22]. Subsequent reports detailing the fractionation and partial purification of the cyclin ubiquitination components have used either cyclin fragments or fusion proteins to assay for these components [12,13,23]. While it is clear that cyclin protein sequences can be ubiquitinated, it is unclear whether ubiquitin initiates proteolysis of full-length cyclin or whether a Ub-independent proteolytic cleavage destabilizes cyclin allowing the Ub system to engage and destroy the resulting fragments.

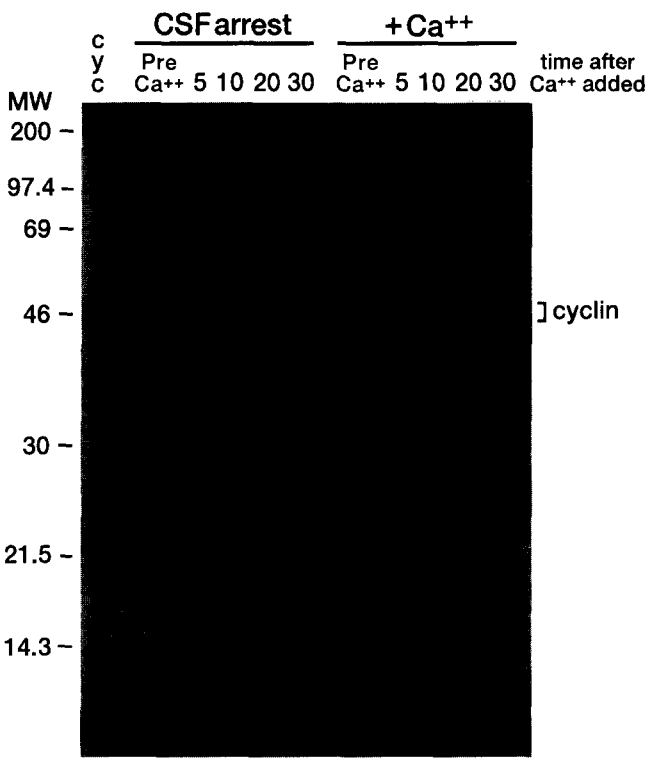

Fig. 1. Degradation of in vitro translated cyclin B2 in *Xenopus* egg extract. CSF arrested extract was prepared as described in section 2, cycloheximide was added to a final concentration of 0.1 mg/ml and incubated for 5 min at 23°C. Extract containing in vitro translated [^{35}S]cyclin was diluted 1:10 into the CSF arrested extract and held for 10 min at 23°C. The time course shown (minutes) was initiated by adding $CaCl_2$ to 0.4 mM, or as a dilution control, H_2O was added to maintain CSF arrest. At the indicated times 3 μl of extract was removed, quenched in SDS-sample buffer and analyzed by SDS-PAGE followed by autoradiography. The lane labeled 'cyc' shows untreated [^{35}S]cyclin. Molecular mass markers are indicated on the left. The bracket denotes the position of [^{35}S]cyclin B2.

To produce a sensitive and physiologically relevant system to study cyclin ubiquitination, we synthesized full-length, [^{35}S]methionine labeled *Xenopus* cyclin B2 by in vitro translation in nuclease treated *Xenopus* egg extract. The in vitro translated cyclin is an ideal substrate by several criteria: it is the physiologic substrate from the same species, thus avoiding complications from a heterologous system, it can be metabolically labeled with [^{35}S]methionine thereby eliminating potential proteolysis artifacts from other labeling procedures [24], and it is the only labeled protein in the extract, thereby facilitating detection of Ub conjugates. Furthermore, synthesis in *Xenopus* egg extract rather than reticulocyte lysate precludes introduction of reticulocyte enzymes in the degradation assay. This cyclin is also preferable to cyclin expressed in bacteria where the full-length product is insoluble, necessitating denaturing conditions during purification that could affect its substrate characteristics. Lastly, the in vitro translated cyclin effectively mimics the substrate characteristics of endogenous cyclin B2. Cyclin is relatively stable in CSF arrested extract, yet is rapidly and completely destroyed when Ca^{2+} is added to induce cell cycle progression. This is evident from the autoradiograms presented in Fig. 1. In all experiments, histone H1 kinase activ-

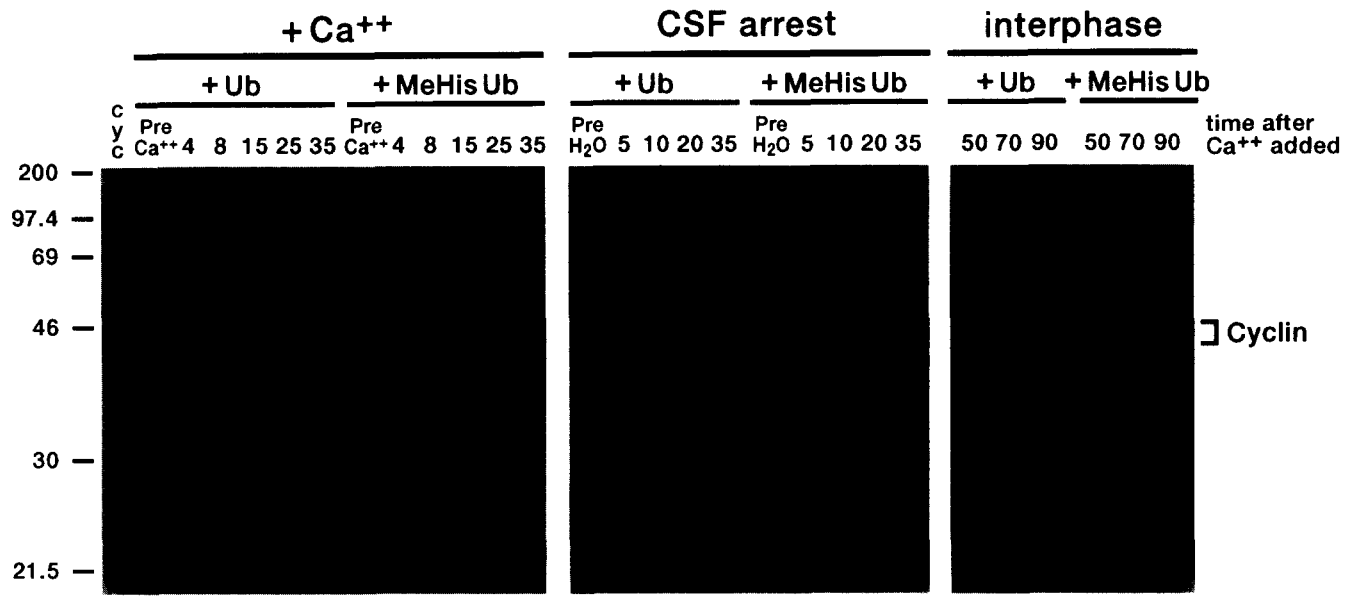

Fig. 2. Effects of Ub or MeHisUb on cyclin stability in 3 cell-cycle phases. CSF extract was prepared and cycloheximide treated as described in Fig. 1 legend. The order of manipulations for '+Ca^{2+}' and 'CSF arrest' lanes was as follows: Extract containing ^{35}S-labeled cyclin was diluted 1:10 into CSF arrested extract 20 min before $CaCl_2$ addition; Ub or MeHisUb was added to a final concentration of 1.6 mg/ml, 10 min before $CaCl_2$ addition; $CaCl_2$ was added to 0.4 mM or H_2O was added as a dilution control in 'CSF arrest' to begin the time course shown (minutes). For 'interphase' extract, $CaCl_2$ was added to 0.4 mM; after 30 min a 1/10 volume of ^{35}S-labeled cyclin was added; after 40 min either Ub or MeHisUb were added to 1.6 mg/ml. At the indicated times, samples were removed and processed as described in the legend for Fig. 1. Molecular mass markers are shown on the left. Untreated cyclin is shown in the lane marked 'cyc'. The bracket denotes the position of [^{35}S]cyclin B2.

ity was measured as an independent means of monitoring the cell-cycle stage of the extract (data not shown).

In the experiment shown in Fig. 1, we could not visualize any higher molecular weight species of cyclin corresponding to Ub conjugates. In order to optimize the sensitivity for detecting ubiquitinated cyclin species, we added methylated 6-his-tagged Ub (MeHisUb) to the extract and monitored its effect on cyclin degradation. As a control, an equal concentration of unmodified Ub was added. In the experiment shown in Fig. 2, the effects of MeHisUb on cyclin degradation were examined in three different cell-cycle phases. Cyclin is relatively stable in both CSF arrested and interphase extract (half life ~65 min). However, cyclin is rapidly degraded when Ca^{2+} is added to release the CSF arrest (half life ~8 min). MeHisUb partially inhibits cyclin proteolysis and results in a marked accumulation of MeHisUb-cyclin conjugates. Unmodified Ub also resulted in a modest accumulation of cyclin conjugates, probably due to isopeptidase inhibition (see below).

The levels of cyclin-Ub conjugates are determined by a balance between the rates of conjugate formation, disassembly by isopeptidases and proteolysis. When the system is unperturbed, cyclin-Ub conjugates are not detected (Fig. 1). The equilibrium can be shifted to reveal cyclin-Ub conjugates by simply adding MeHisUb or unmodified Ub. We believe that both reagents exert these effects by partially inhibiting isopeptidases rather than stimulating conjugation because addition of C-terminal Ub peptide extensions also results in conjugate accumulation (unpublished data). The more dramatic effects observed in the presence of MeHisUb indicate that it is either a more potent isopeptidase inhibitor than Ub or that blocking chain extension reactions shifts the equilibrium towards conjugate accumulation. The His-tag itself has minimal impact on this equilibrium shift, as the same effects are seen when MeUb is added to the system (data not shown).

Two lines of evidence indicate that the observed higher molecular weight species are Ub conjugates of cyclin. First, cyclin is the predominant labeled band so the shifted bands must have originated from cyclin. Second, the 6-histidine tag on MeHisUb imparts a slight mobility shift to proteins conjugated to it. As shown in Fig. 3, cyclin conjugates formed from MeHisUb migrate slightly slower on SDS PAGE than conjugates formed from unmodified Ub. Furthermore, the MeHisUb conjugates of cyclin can be purified with a nickel affinity column (data not shown).

The data presented in Fig. 2 show that MeHisUb results in an accumulation of cyclin-MeHisUb conjugates. During the degradation phase (+Ca^{2+}), conjugates are most prominent and cyclin proteolysis is partially inhibited. These results are consistent with the idea that ubiquitination regulates rapid cyclin proteolysis. However, because MeHisUb only partially inhibits cyclin proteolysis, it is possible that dual pathways exist for cyclin degradation.

An apparent paradox is also evident from the results in Fig. 2. Ubiquitinated cyclin is observed in CSF arrested and interphase extract, even though cyclin is not rapidly degraded. Presumably, MeHisUb alters the existing equilibrium to result in conjugate buildup, indicating that a low, normally undetectable, level of cyclin conjugation occurs throughout the cell-cycle. This finding is consistent with the results of Glotzer et al. [10] who also observed a low level of ubiquitination during interphase. Interestingly, quantitation of the gels shown in Fig. 2 show that cyclin's half life during CSF arrest and interphase is not altered by addition of MeHisUb (data not shown). Although this might suggest that these conjugates are not prote-

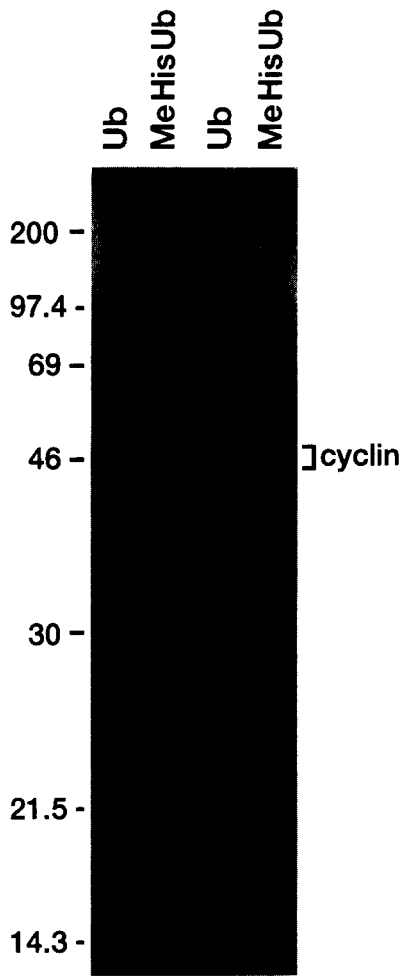

Fig. 3. Electrophoretic mobility comparison of cyclin conjugates formed with either MeHisUb or unmodified Ub. CSF arrested extract was prepared and manipulated exactly as described in Fig. 2 for '+Ca^{2+}' extract. The reactions were quenched 5 minutes after $CaCl_2$ addition. Extract samples containing either Ub or MeHisUb were loaded in alternating lanes and subjected to SDS-PAGE and autoradiography. Molecular mass markers are indicated on the left and the bracket on the right denotes unconjugated [^{35}S]cyclin B2.

olytic intermediates, MeHisUb may be a less effective inhibitor when the half life of cyclin is 65 min rather than 8 min.

Our results indicate that full-length cyclin B2 is a substrate for the Ub system and that prior proteolytic processing is not a prerequisite for ubiquitination. The observed cyclin-Ub conjugates are approximately the expected molecular weight for full-length conjugates. However, drawing firm conclusions from the apparent molecular weight of cyclin conjugates is difficult; cyclin phosphorylation results in a significant gel mobility shift [25] and defining absolute molecular weights for Ub conjugate ladders has historically been problematical [26]. Therefore, the observed conjugates are consistent with ubiquitination of the full-length protein, but from this observation alone we cannot rule out removal of several amino acids from either end of cyclin. To be significant in destabilizing cyclin, the putative proteolytic event would be expected to occur only during the cyclin degradation phase. Because we observe cyclin conjugates during CSF arrest and interphase, the most likely explanation is that full-length cyclin is ubiquitinated.

In summary, Ub conjugates of full length cyclin can be detected. This finding adds further support for the involvement of the Ub pathway in cyclin degradation. However, we also can detect cyclin conjugates independent of rapid cyclin proteolysis implying that the regulation of cyclin destruction may be more complex than simply coupling Ub to cyclin. Lastly, our results indicate that full-length cyclin is ubiquitinated, lessening the possibility that a prior proteolytic attack targets cyclin fragments for ubiquitination.

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