Cleavage of cyclin A at R70/R71 by the bacterial protease OmpT

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Previous work has shown that cyclin A can be cleaved at Arg-70/Arg-71 by a proteolytic activity present in an *in vitro*-coupled transcription/translation system by using rabbit reticulocyte lysate programmed by plasmid DNA encoding p27^{KIP1}, a cyclin-dependent kinase inhibitor, but not by plasmid DNAs encoding other cyclin-dependent kinases inhibitors. Here we report that cyclin A is also cleaved by translation product programmed by plasmid DNA encoding cyclin B. Several findings indicate that the cleavage activity in this assay is provided by the bacterial protease OmpT, which cofractionates with cyclin B and p27^{KIP1} plasmid DNAs and is thus carried over into the coupled *in vitro* transcription/translation reactions. (*i*) Cleavage activity appeared even when transcription or translation of the cyclin B or p27^{KIP1} was blocked. (*ii*) Activity resembling OmpT, a serine protease that cleaves between dibasic residues, routinely copurifies with p27^{KIP1} and cyclin B plasmid DNAs. (*iii*) Both cyclin A cleavage activity and OmpT activity are heat stable, resistant to denaturation, and inhibited by Zn^{2+}, Cu^{2+}, or benzamidine. (*iv*) Cyclin A cleavage activity is detected when using lysates or DNAs prepared from *Escherichia coli* strains that contained OmpT but not with strains lacking OmpT. (*v*) Purified OmpT enzyme itself cleaves cyclin A at R70/R71. These data indicate that OmpT can be present in certain DNA preparations obtained by using standard plasmid purification protocols, and its presence can potentially affect the outcome and interpretation of studies carried out using *in vitro*-translated proteins.

coupled transcription/translation | cell cycle | p27^{KIP1}

Cyclin-dependent kinases (CDKs) are key regulators of the eukaryotic cell cycle whose activities are tightly regulated by phosphorylation and interactions with regulatory subunits (1, 2). Activation of CDKs involves association with a cyclin subunit and phosphorylation at Thr-161. The activity of CDKs can be inhibited by phosphorylation at Thr-14 and Tyr-15, by binding to CDK inhibitors, and by proteolytic degradation of the cyclin subunit. Degradation of mitotic cyclins A and B at the end of mitosis requires a conserved mitotic destruction box motif near the N terminus, which acts as a signal for ubiquitin-dependent proteolysis (3–6).

Cyclin A is also the target of other proteases. When *Xenopus* embryos are treated with hydroxyurea under conditions that induce apoptosis, cyclin A2 is cleaved by IL-1β-converting enzyme-like caspases at $D^{87}EPD^{90}\downarrow$ (equivalent to $D^{104}EAE^{107}$ in human cyclin A2) (7). This generates a truncated cyclin A that lacks the mitotic destruction box and thus is predicted to be stable. *In vitro*, rabbit reticulocyte lysate programmed by coupled transcription/translation of plasmid DNA encoding the CDK inhibitor p27^{KIP1} induces proteolytic cleavage of cyclin A downstream of the destruction box, at or very close to R70/R71, yielding a truncated cyclin A that was shown to be stable. Only translation products programmed by p27^{KIP1} DNA, but not by other CDK inhibitors, induce this cleavage (8). Here we show that, like p27^{KIP1}DNA, reticulocyte lysate programmed by cyclin B DNA also induces cleavage of cyclin A at R70/R71. Several results now indicate that this cleavage activity is not induced by the cyclin B or p27^{KIP1} proteins themselves, but is due, instead, to a bacterial protease, OmpT, which copurifies with these two plasmid DNA when obtained by using routine procedures for preparing plasmid DNA.

Materials and Methods

DNA Constructs. All cyclin A and cyclin B constructs used in this study encoded human cyclin A2 and human cyclin B1, respectively. Unless otherwise indicated, all of the plasmid DNAs used for coupled *in vitro* transcription/translation reactions were prepared by using DH5α cells. Cyclin A in pET21d, FLAG-cyclin A in pUHD-P1, and glutathione *S*-transferase (GST)-cyclin A in pGEX-KG were as described (9). GST-cyclin A(CΔ114) in pGEX-KG was constructed by cutting GST-cyclin A with *Sal*I and *Pvu*I (partial), treated with Klenow enzyme, followed by ligation (the product contained an extra 15 amino acids cloning artifact at the C terminus). GST-cyclin A(CΔ70) in pGEX-KG was made as CΔ114 except that *Pst*I was used instead of *Pvu*I. Site-directed mutagenesis of R70A + R71A was constructed as described (10), using the oligonucleotide 5′-GGCCGAAGACTGCAGCTGTTGCACCCCT-3′ and its antisense to introduce the mutation. Cyclin B in pET21d was as described (9). The *Nco*I–*Xho*I fragment of cyclin B was first put into pGEX-KG. GST-cyclin B in pGEX-KG was then cut with *Kpn*I–*Sal*I, treated with Klenow enzyme, and religated [GST-cyclin B(CΔ85) in pGEX-KG]; the *Nco*I–*Xho*I fragment was then put into pET21d [cyclin B(CΔ85) in pET21d]. GST-cyclin B in pGEX-KG was amplified with 5′-GTACCCATGGTGGTGCCAGTGCC-3′ and pGEX reverse primers, cut with *Nco*I–*Xho*I, and ligated into pET21d [cyclin B(NΔ88)-H6 in pET21d]. p27 in pET21a was as described (11). Plasmid DNA was prepared from different strains of *Escherichia coli* by using the Qiagen midi- or maxi-DNA purification columns (Qiagen, Hilden, Germany), and the Wizard plus minipreps DNA purification system (Promega) according the manufacturers' instructions. In some experiments, OmpT was amplified from DH5α by PCR with the primers 5′-GGCCATGGGGGCGAAACTTCTGGGA-3′ and 5′-GCTCGAGAAATGTGTACTTAAGACCAG-3′. The PCR product was cleaved with *Nco*I and *Xho*I and ligated into pET21d (to make OmpT-H6 in pET21d). In other experiments, OmpT plasmid DNA was a gift from Nick Decker (Utrecht University, Utrecht, The Netherlands).

Cell Culture. HtTA1 cells are HeLa cells (human cervical carcinoma) stably transfected with pUHD15–1 expressing the tTA tetracycline repressor chimera. Cell growth and transfection were as described (9). Cell extracts were prepared with hypotonic

Abbreviations: CDK, cyclin-dependent kinase; RL, reticulocyte lysate; GST, glutathione *S*-transferase.

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buffer (9) for destruction assays or with Nonidet P-40 buffer (11) for immunoprecipitation.

Expression and Purification of Recombinant Proteins. Coupled transcription-translation reactions in the presence of [^{35}S]methionine in rabbit reticulocyte lysate RL were performed according to the manufacturer's instructions (Promega), using the indicated plasmid DNAs (1/10 vol of 1 mg/ml). Expression of GST-tagged and histidine-tagged proteins in *E. coli* strain BL21(DE3) and purification with glutathione agarose and Ni-nitrilotriacetic acid agarose chromatography, respectively, were as described (12).

Cyclin A Cleavage Assays. Purified bacterially expressed cyclin A (1–5 μg in 1 μl), RL produced cyclin A (1 μl), or cyclin A immunoprecipitates (10 μl) were mixed with 1 μl of RL-produced cyclin B and 8 μl (or 18 μl for immunoprecipitates) of hypotonic buffer. The mixtures were incubated at 37°C for the indicated time and mixed with 20 μl of SDS sample buffer. The samples were then analyzed by SDS/PAGE or Tricine gel and followed by immunoblotting, autoradioagraphy, or Coomassie blue staining as described (13). The bacterial extracts used for cleavage assays were prepared with a lysozyme lysis method as described (12). Approximately 200 μl of lysate was produced from 1 ml of bacteria suspension, and 2 μl of the lysate was used for the cyclin A cleavage assay. Protease inhibitors were used at the following concentration: benzamidine (5 mM), E64 (10 μM), EDTA (5 mM), leupeptin (100 μM), pepstatin (1 μM), phenylmethylsulfonyl fluoride (PMSF) (1 mM), and soybean trypsin inhibitor (2.5 μg/ml).

Antibodies and Immunological Methods. mAbs against FLAG tag (M2) and against PSTAIRE were as described (9). Rabbit anti-FLAG polyclonal antibodies were gifts from K. Yamashita (Kanazawa University, Kanazawa, Japan) or from Santa Cruz Biotechnology (sc-807). Anti-cyclin A mAb E23 was a gift from T. Hunt (Imperial Cancer Research Fund, South Mimms, U.K.). Immunoblotting and immunoprecipitation were performed as described (11).

Results

RL Programmed by Cyclin B Plasmid DNA Induces Cleavage of Cyclin A Between R70 and R71. During the course of experiments investigating the degradation of cyclin A and B, we observed that a bacterially expressed GST fusion protein containing the N-terminal destruction box of cyclin A (CΔ114, containing residues 1–114 of cyclin A) was cleaved into a smaller product (with a loss of ≈10 kDa) when incubated with rabbit RL programmed to produce cyclin B [cyclin B(RL)], using cyclin B plasmid DNA and coupled *in vitro* transcription/translation (Fig. 1*A*). Unprogrammed RL did not induce cleavage of cyclin A (lane 3).

Cyclin A cleavage was seen most readily with GST-cyclin A(CΔ114), but full-length cyclin A produced in mammalian cells (Fig. 1*B*) or in RL (Fig. 2*A*) also could be cut when incubated with cyclin B(RL). Full-length cyclin A was cleaved into two fragments of ≈50 kDa and ≈10 kDa on SDS/PAGE (Fig. 1*B*). The 10-kDa fragment contained the N terminus because it was recognized by antibody M2 against the N-terminal FLAG epitope. The 50-kDa fragment contained the C terminus because it was not detected by M2, but by E23 anti-cyclin A antibody, which epitope was mapped to the C-terminal half of cyclin A (data not shown).

The sizes of the cyclin A cleavage products were consistent with the cleavage site being close to R70, the site of cleavage induced by p27^{KIP1} translation product (8). N-terminal sequencing of the larger fragment yielded the sequence RVAPLKDLPVNDEHV, which perfectly matches the sequence of cyclin A starting from R71. Initial comparisons of cleavage specificity

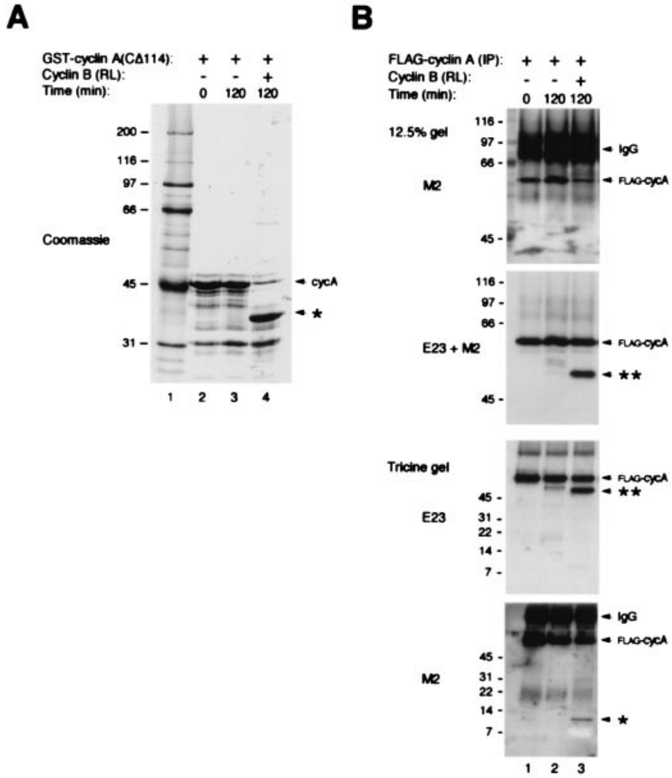

Fig. 1. Cleavage of cyclin A by cyclin B translated in rabbit RL. (*A*) Cyclin B(RL) induces efficient cleavage of cyclin A(CΔ114). GST-cyclin A(CΔ114) was mixed with unprogrammed RL (lanes 2 and 3) or cyclin B(RL) (lane 4). The reactions were incubated at 37°C and stopped with SDS-sample buffer at the indicated time. The proteins were analyzed by SDS/PAGE and Coomassie blue staining. Molecular size standards (lane 1) in kDa are indicated on the left. The positions of GST-cyclin A(CΔ114) and the cleaved form of cyclin A (*) are indicated on the right. (*B*) Cleavage of cyclin A expressed in mammalian cells into two fragments by cyclin B(RL). FLAG-cyclin A was transiently transfected into HtTA1 cells. Cell extracts were prepared and the expressed cyclin A were immunoprecipitated with anti-FLAG antibodies. The immunoprecipitates were incubated with unprogrammed RL or cyclin B(RL) as indicated. The samples were applied onto 12.5% SDS/PAGE (top two panels) or Tricine gel (bottom two panels), and subjected to immunoblotting with anti-FLAG mAb M2, anti-cyclin A monoclonal E23, or M2 and E23 together as indicated. In this paper, the N-terminal fragment of cyclin A is denoted with "*", and the C-terminal fragment is denoted with "**" (see main text).

were carried out, as shown in Fig. 2*A*. Reticulocyte translation product programmed by the addition of cyclin A plasmid DNA [cyclin A(RL)] yielded a single major radiolabeled band and no obvious cyclin A cleavage products (lanes 8 and 9). The addition of translation product programmed by the addition of cyclin B plasmid DNA [cyclin B(RL)] to cyclin A(RL) led to the appearance of a cleaved cyclin A fragment (lane 10). By contrast, cyclin A mutated at R70 and R71 (R70A + R71A) was not cleaved after incubation with cyclin B(RL) (lane 6). The proteasome inhibitor LLnL failed to inhibit cyclin A cleavage (lane 11). Similarly, mammalian cell-expressed FLAG-cyclin A, but not the R70A + R71A mutant, was cleaved by cyclin B(RL) (Fig. 2*B*). Taken together, these data indicated that cyclin A was cleaved between R70 and R71 by a proteolytic activity present in cyclin B(RL). Thus, the properties of cleavage induced by cyclin B(RL) were very similar to those previously described for cleavage induced by p27^{KIP1}(RL).

The *in Vitro* Cleavage of Cyclin A Is Attributable to a Bacterial Protease That Copurifies with p27^{KIP1} and Cyclin B Plasmid DNAs. That both cyclin B and p27^{KIP1} *in vitro* RL translation mixes were capable

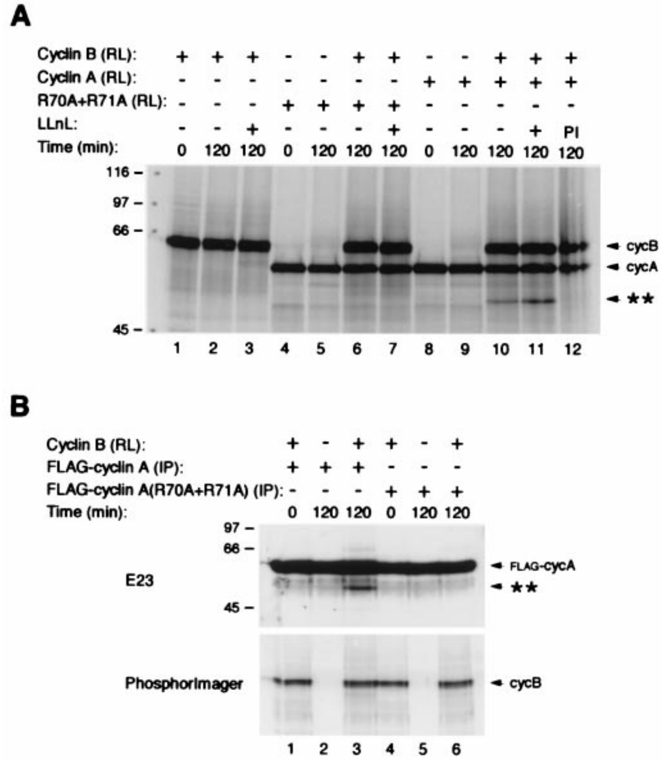

Fig. 2. Cyclin A cleavage is abolished by R70A + R71A mutation. (*A*) RL expressing cyclin B, cyclin A, or cyclin A(R70A + R71A) were mixed as indicated. LLnL (50 μM) or protease inhibitor (PI) mixture was included in the reactions where indicated. The reactions were incubated for the indicated time before stopped with SDS sample buffer. The samples were analyzed by SDS/PAGE and phosphorimagery. The positions of the molecular size standards (in kDa), the expressed proteins, and the cleaved form of cyclin A (**) are indicated. (*B*) FLAG-cyclin A (lanes 1–3) or R70A + R71A mutant (lanes 4–6) were transiently transfected into HtTA1 cells. Cell extracts were prepared, and the expressed cyclin A were immunoprecipitated with anti-FLAG antibodies. The immunoprecipitates were incubated with unprogrammed RL or cyclin B(RL) as indicated. Cleavage of cyclin A was analyzed by immunoblotting with anti-cyclin A mAb E23 (*Upper*), and expression of cyclin B was analyzed with phosphorimagery (*Lower*).

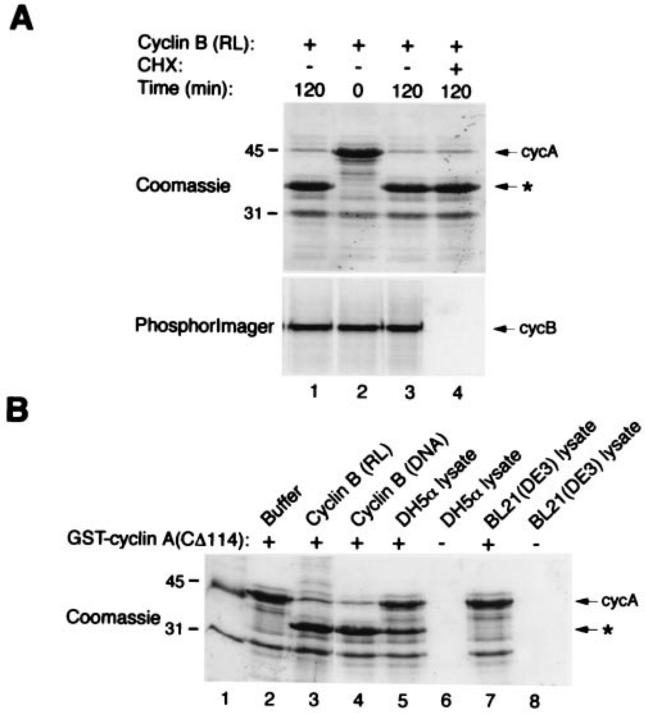

Fig. 3. RL-translated cyclin B protein is not required to induce cyclin A cleavage. (*A*) Blocking cyclin B synthesis does not affect cyclin A cleavage. Cyclin B DNA was mixed with RL either in the absence or presence of cycloheximide (1 mg/ml). No cyclin B translation was detected when cycloheximide was added (*Lower*). The RL were then incubated with GST-cyclin A(CΔ114) for the indicated time, and cleavage of cyclin A was detected by SDS/PAGE and Coomassie blue staining (*Upper*). (*B*) Cyclin A proteolytic activity is present in the DNA and DN5α lysate. GST-cyclin A(CΔ114) was incubated with buffer, cyclin B(RL), cyclin B DNA, DH5α lysate, or BL21(DE3) lysate as indicated. GST-cyclin A(CΔ114) was not added in the reactions in lanes 6 and 8. Cleavage of cyclin A was detected by SDS/PAGE and Coomassie blue staining. Molecular size standards (lane 1) in kDa are indicated on the left.

of cleaving cyclin A at R70/R71 was unexpected, and led us to reexamine the original idea that these proteins were capable of activating a protease present in RL. Additional observations (data not shown) added to our suspicion of a different explanation. (*i*) Unlike cyclin B or p27^{KIP1} produced by *in vitro* translation, neither bacterially expressed versions of these proteins nor cyclin B or p27^{KIP1} immunoprecipitated from mammalian cells induced cleavage. (*ii*) Cyclin B(RL) was not able to activate more proteolytic activity in unprogrammed RL. (*iii*) Blocking the kinase activity of cyclin B-CDK with butyrolactone I did not affect cleavage. (*iv*) No cleavage of the endogenous or transfected cyclin A was observed when cyclin B or p27^{KIP1} was cotransfected into HeLa cells. (*v*) Both cyclin B(CΔ85) and cyclin B(NΔ88) expressed in RL could induce cleavage of cyclin A, suggesting that neither any unique region of cyclin B or the ability to activate CDK was important for the cleavage. Taken together, these results suggested that factors other than the *in vitro*-translated cyclin B or p27^{KIP1} proteins themselves might be responsible for the observed cleavage activity.

An important clue was provided by the finding that the addition of cycloheximide to block synthesis of cyclin B during *in vitro* translation did not block the formation of cyclin A cleavage activity (Fig. 3*A*). Furthermore, the addition of cyclin B plasmid DNA alone to recombinant cyclin A protein, i.e., in the absence of any RL, was sufficient to induce cyclin A cleavage (Fig. 3*B*, lane 4). Because only cyclin A protein and cyclin B DNA were present in that reaction, these results raised the possibility that cyclin A-cleaving activity was intrinsic to the DNA preparation. Moreover, lysates of DH5α cells alone (the host cells used for cyclin B plasmid DNA preparation) contained an activity that could cleave cyclin A (Fig. 3*B*, lane 5). The proteolytic activity was not affected by whether the DH5α has been transformed with plasmid DNA (data not shown).

Similarly, robust cyclin A cleavage activity was present in RL programmed by the addition of p27^{KIP1} plasmid DNA, even when transcription or translation was blocked by omission of RNA polymerase or addition of cycloheximide, respectively (Fig. 4*A*). Addition of p27^{KIP1} plasmid DNA alone (when prepared from DH5α cells) to HeLa cell lysate also induced cleavage of endogenous cyclin A (Fig. 4*B*, lanes 1 and 2). Finally, phenol extraction of p27^{KIP1} plasmid DNA before *in vitro* transcription/translation removed cleavage activity (data not shown). Taken together, these results argued strongly that standard preparations of cyclin B and p27^{KIP1} plasmid DNAs (but not cyclin A plasmid DNA, see Fig. 4*A*, lanes 1 and 2) contained a protease that presumably copurified with those plasmid DNAs and that this protease activity was responsible for cleavage of cyclin A at R70/R71.

Clues about the identity of the protease were provided by the observations that (*i*) lysates of DH5α cells but not BL21(DE3) cells cleaved cyclin A at R70 (Fig. 3*B*) and (*ii*) DNA prepared from DH5α, but not BL21 cells, cleaved cyclin A (Fig. 4*B*). This

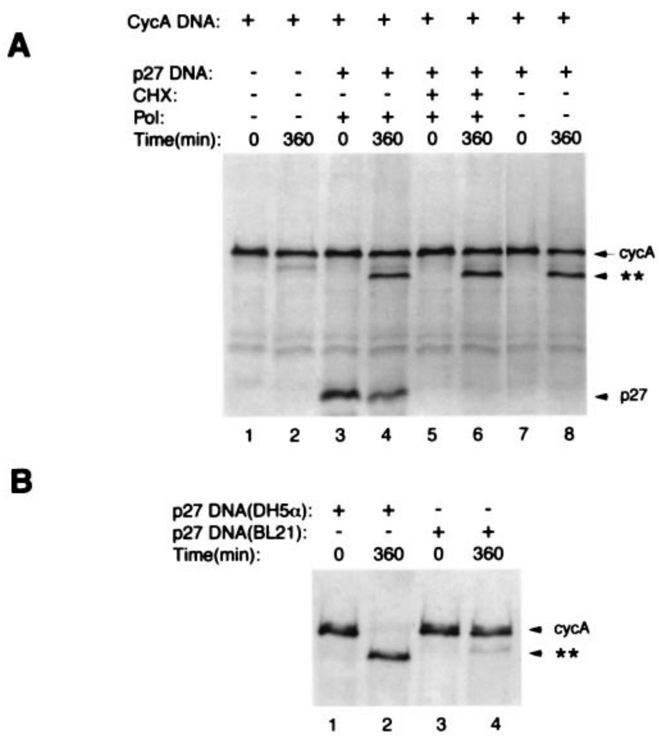

Fig. 4. RL-translated p27KIP1 is not required to induce cyclin A cleavage. (*A*) Cyclin A translation product was produced by the addition of cyclin A plasmid DNA to the RL-coupled transcription/translation system. p27KIP1 plasmid DNA was added to coupled transcription/translation RL in the absence or presence of cycloheximide and RNA polymerase as indicated. Reactions were mixed and incubated as indicated and analyzed by SDS/PAGE followed by autoradiography. (*B*) p27KIP1 plasmid DNA was prepared from *E. coli* strain DH5α (lanes 1 and 2) and BL21 (lanes 3 and 4). The plasmid DNA preps were mixed with cyclin A(RL) for the indicated time.

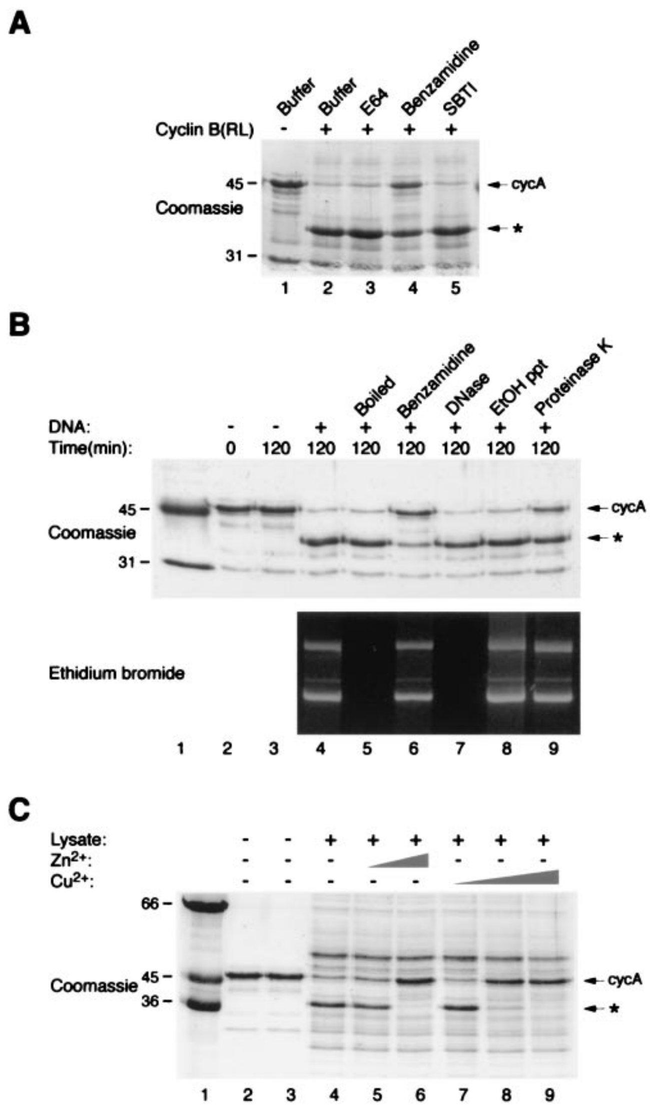

Fig. 5. Cyclin A proteolytic activity is present in DNA preparation and in bacterial lysates. (*A*) Inhibition of cleavage of cyclin A by benzamidine. GST-cyclin A(CΔ114) was incubated with cyclin B(RL) in the presence of buffer (lane 2), E64 (lane 3), benzamidine (lane 4), or soybean trypsin inhibitor (lane 5) at 37°C for 120 min. Cyclin A cleavage was detected by SDS/PAGE and Coomassie blue staining. Molecular size standards in kDa are indicated on the left. (*B*) Cyclin B in pET21d DNA (lanes 4–9) were boiled for 5 min (lane 5), subjected to ethanol precipitation (lane 8), or treated with benzimidine (lane 6), DNase (lane 7), or proteinase K (lane 9). The proteinase K was subsequently inactivated by phenol/chloroform extraction. The samples were then incubated with purified GST-cyclin A(CΔ114) for the indicated time. Cleavage of cyclin A was detected by SDS/PAGE and Coomassie blue staining (*Upper*). Molecular size standards (lane 1) in kDa are indicated on the left. DNA in the samples was visualized by agarose gel electrophoresis and ethidium bromide staining (*Lower*). (*C*) GST-cyclin A(CΔ114) was incubated with buffer (lanes 2 and 3), or DH5α lysates (lanes 4–9) in the presence of $ZnCl_2$ (0.1 mM, lane 5; 1 mM, lane 6) or $CuCl_2$ (0.1 mM, lane 7; 1 mM, lane 8; 10 mM, lane 9). Cleavage of cyclin A was detected by SDS/PAGE and Coomassie blue staining. Molecular size standards (lane 1) in kDa are indicated on the left.

suggested that the protease was present in DH5α but not BL21(DE3) strains of *E. coli*. Comparison of the genotypes of these strains revealed a promising candidate, OmpT (EC 3.4.21.87), which cleaves between dibasic residues (14, 15), and is present in DH5α but not BL21(DE3) cells.

OmpT is heat stable and is active even under extreme denaturing conditions (16) but is inhibited by benzamidine, Zn^{2+}, and Cu^{2+} (17). Among the protease inhibitors tested [benzamidine, E64, EDTA, leupeptin, *N*-acetyl-L-leucinyl-L-leucinyl-L-norleucinal (LLnL), pepstatin, and soybean trypsin inhibitor (SBTI)], only benzamidine showed strong inhibition of cyclin A cleavage (Fig. 5*A*); phenylmethylsulfonyl fluoride showed weak inhibition (data not shown). Cleavage activity associated with cyclin B plasmid DNA preparations was not denatured by alkali treatment, boiling, ethanol precipitation, or DNase treatment (Fig. 5*B*) but was inhibited by Zn^{2+} and Cu^{2+} (Fig. 5*C*). Taken together, these data suggested that cleavage of cyclin A R70/R71 induced by RL-expressed cyclin B and p27KIP1 was attributable to an OmpT-like bacterial protease that copurified with the cyclin B and p27KIP1 plasmid DNAs used to program the translation reactions.

***E. Coli* OmpT Can Cleave Cyclin A.** To test whether OmpT might be responsible for the cleavage of cyclin A, we expressed OmpT in *E. coli* strains that originally lacked the OmpT gene. As seen above, neither extracts of BL21(DE3), which does not contain OmpT (Fig. 3*B*), nor extracts of BL21(DE3) transformed with cyclin B DNA (Fig. 6*A*) contained any cyclin A cleavage activity. By contrast, cyclin A cleavage activity was present in extracts of BL21(DE3) transformed with an OmpT-expressing construct (Fig. 6*A*). We next expressed histidine-tagged OmpT (OmpT-H6) in BL21(DE3) and purified OmpT-H6 with Ni-agarose chromatography. Fig. 6*B* shows that purified OmpT-H6 cleaved cyclin A into a smaller product of similar size as cleavage at R70. The R70A + R71A mutant of cyclin A was not cleaved by OmpT-H6 (data not shown).

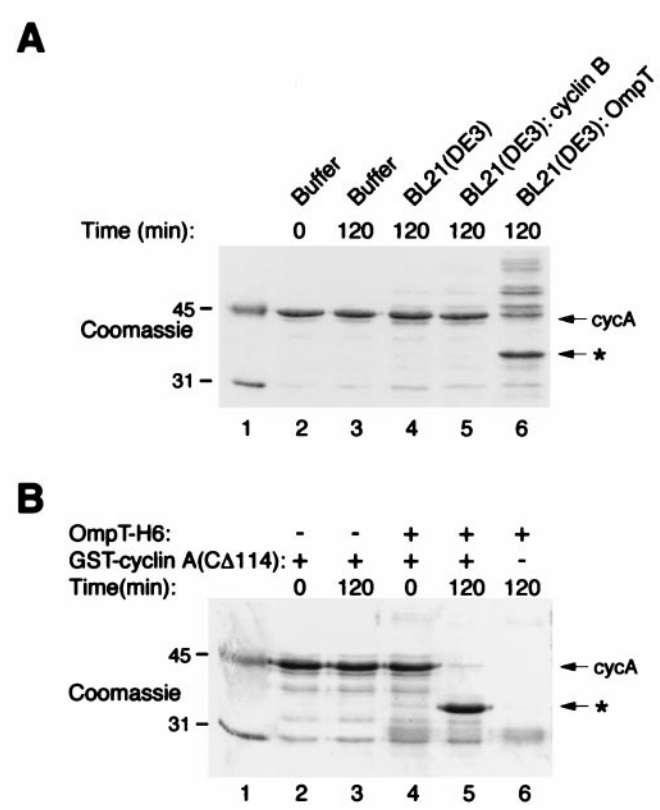

Fig. 6. Cleavage of cyclin A by OmpT. (*A*) GST-cyclin A(CΔ114) was incubated with buffer (lanes 2 and 3), lysates of BL21(DE3) (lane 4), BL21(DE3) transformed with cyclin B construct (lane 5), or transformed with OmpT expression construct (lane 6) for the indicated time. Cleavage of cyclin A was detected by SDS/PAGE and Coomassie blue staining. Molecular size standards (lane 1) in kDa are indicated on the left. (*B*) Purified OmpT protein (lanes 4–6) was incubated with GST-cyclin A(CΔ114) (lanes 2–5) for the indicated time. Cleavage of cyclin A was detected by SDS/PAGE and Coomassie blue staining. Molecular size standards (lane 1) in kDa are indicated on the left.

Taken together, these observations argue that cyclin A-cleaving activity is not attributable to a RL protease that is activated in response to p27^{KIP1} or cyclin B translation product but is, instead, attributable to OmpT, a bacterial protease that copurifies with the cyclin B and p27^{KIP1} plasmid DNAs used to program the coupled transcription/translation reactions.

Discussion

This study was originally initiated to see whether cyclin B affects the degradation of cyclin A, given that cyclin A is degraded slightly early than cyclin B in the cell cycle (18). The finding that cyclin B(RL) could induce the cleavage of cyclin A at R70/R71 seemed problematic because cleaved cyclin A lacks the mitotic destruction box and is thus be expected to resist degradation during exit from mitosis. Furthermore, the idea that both cyclin B and p27^{KIP1} activated the same pathway leading to cyclin A cleavage did not fit easily into any current model of cell-cycle regulation. The results presented here now explain these effects: the ability of cyclin B and p27^{KIP1} *in vitro* translation product to cleave cyclin A is attributable to the presence of the bacterial protease OmpT, which copurifies with the plasmid DNAs that are used to drive synthesis of the cognate proteins in the coupled *in vitro* transcription/translation systems. Curiously, OmpT activity routinely copurified with cyclin B and p27^{KIP1} plasmid DNA but was rarely detected at any significant levels with cyclin A or other CDK inhibitor plasmid DNAs. Perhaps either the amount of protease or the extent of copurification with different plasmid DNAs is somehow influenced by DNA sequence or the expression levels. At present, we have no explanation for this.

Cleavage activity was present in DNAs prepared using different matrix-based methods, including several different commercial plasmid DNA purification kits and noncommercial methods using glass bead matrices. Coupled transcription/translation reaction products programmed by DNAs made using these methods are frequently used to generate radiolabeled or tagged proteins that are used to assay the functional properties of wide range of proteins. Although phenol extraction readily removes OmpT activity and other proteins from these plasmid DNAs, this step is usually not included. Clearly, its omission has the potential to affect the outcome and interpretation of studies carried out using *in vitro*-translated proteins.

Smaller forms of cyclin A resembling the cleaved form described here have been detected *in vivo* in FR3T3 cells, 293 cells and other mammalian tissue culture cells (8), suggesting that the R70/R71 region might be susceptible to cleavage by a mammalian protease resembling OmpT. The possibility that this sequence might be part of an exposed region also may be significant for structural studies of the N-terminal region of cyclin A, which has so far been remained elusive.

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