Volume 306, number 1, 90–93 FEBS 11275 July 1992

Cyclin A-Cys41 does not undergo cell cycle-dependent degradation in *Xenopus* extracts

Thierry Lorca[a], Alain Devault[a], Pierre Colas[b,c], André Van Loon[b,c], Didier Fesquet[a], Jean Bernard Lazaro[a] and Marcel Dorée[a]

[a]*CNRS UPR 8402 and INSERM U 249, 1919 route de Mende, BP 5051, 34033 Montpellier Cedex, France,* [b]*Ecole Normale Superieure, Laboratoire de Biologie Cellulaire et Moléculaire, 46 Allée d'Italie, 69364 Lyon Cedex 07, France* and [c]*Department of Experimental Zoology, State University of Utrecht, Padualaan 8, 3584 CH Utrecht, The Netherlands*

Received 13 May 1992

Truncated cyclin A and cyclin B lacking the N-terminal domain comprising the 'destruction box' escape from proteolysis and arrest cells at metaphase. Mutation of a conserved arginine residue of the destruction domain makes cyclin B resistant to proteolysis. Here we show that mutation of the same residue also makes cyclin A resistant to proteolysis, in either of two situations in which the cyclin degradation pathway is turned on: (i) in *Xenopus* extracts of activated eggs where the degradation pathway has been permanently turned on by adding a recombinant undegradable cyclin B in which the arginine residue of the destruction box has been substituted by alanine; (ii) in extracts of metaphase II-arrested oocytes after Ca^{2+}-dependent inactivation of the cytostatic factor (CSF).

cdc2; Cell cycle; Cyclin degradation

1. INTRODUCTION

Exit from M phase of the cell cycle requires inactivation of cyclin-cdc2 kinases (see [1,2] for reviews). This inactivation is brought about by dephosphorylation of threonine 161 on the cdc2 subunit [3], which is itself dependent on the proteolytic degradation of the cyclin subunit [4–6]. Two types of mitotic cyclins, A and B, are detected in most cells and both are able to form active complexes with cdc2. Cell cycle-regulated proteolysis of cyclin A and B is thought to be mediated by a ubiquitin-dependent process, since polyubiquitin chain formation was shown to be required for cyclin destruction [7,8]. The N-terminal domain of cyclin B contains a conserved stretch of amino acids (RXALXXI). Mutations within this domain that inhibit ubiquitin conjugation also inhibit degradation [7]. The integrity of the arginine residue was found to be critical for degradation of cyclin B since substitution for cysteine, alanine or serine rendered it resistant to cell cycle-dependent proteolysis [7,9,10].

The aim of the present work was to test the functional equivalence of the RXALXXI consensus sequence, which is also conserved in cyclin A homologs.

Correspondence address: M. Dorée, CNRS UPR and INSERM U 249, 1919 route de Mende, BP 5051, 30433 Montpellier Cedex, France.

2. MATERIALS AND METHODS

2.1. *Egg extracts*

Interphase *Xenopus* extracts were prepared according to Felix et al. [11]. Briefly, unfertilized eggs were collected into MMR/2 (50 mM NaCl, 1 mM KCl, 0.5 mM $MgCl_2$, 0.05 mM Na-EGTA, 2.5 mM Na-HEPES, pH 7.7) and de-jellied with 2% cysteine, pH 7.8, washed four times and then electrically activated. The activated eggs were incubated for 40 min at room temperature and then transferred to cold acetate buffer (100 mM potassium acetate, 2.5 mM magnesium acetate, 10 μg/ml cytochalasin B, 1 mM DTT, 5 mM EGTA, pH 7.2). Excess acetate buffer was removed prior to centrifugation for 10 min at 12,000 × *g*. The cytoplasmic material was collected from the crushed eggs and an ATP-regenerating system, consisting of 10 mM creatine phosphate, 80 μg/ml creatine kinase, 1 mM ATP (final concentrations), was added. After spinning for 60 min at 100,000 × *g*, the supernatant was collected and kept at −70°C.

Extracts from unfertilized eggs were prepared in the same way except that electric activation was omitted.

2.2. *Recombinant cyclin B*

Full-length cyclin B cDNA of the starfish, *Marthasterias glaciatis*, and the mutant cyclin B-Ala31 (described by Lorca et al. [9]) were cloned in the expression vector, pUEXZZB1B2, downstream of the P_L lambda promotor. Both cyclins were produced as fusion proteins containing 219 residues of the serum albumin-binding domains of streptococcal protein-G at its C-terminus. Expression of the fusion proteins was induced by raising the temperature from 30 to 42°C to inactivate the thermo sensitive λ repressor encoded in the vector. The soluble cyclin B fusion proteins were purified from the bacterial extracts by binding to human serum albumin (HSA)-Sepharose, followed by elution in 10 mM HEPES, pH 7.2, containing 0.25 M lithium diiodosalicylate, and desalting in 10 mM HEPES pH 7.2, on a Sephadex G-5 column.

Starfish cyclin B and *Patella* cyclin A were translated in vitro in the presence of [^{35}S]methionine in reticulocyte lysates (Promega) from their respective RNAs. These were obtained by in vitro transcription

Published by Elsevier Science Publishers B.V.

of plasmids pTZcycB [12] and pGEM5cycA [13], using T7 and SP6 RNA polymerase, respectively.

Cyclin B-Ala31 and cyclin A-Cys41 mutants were generated according to an oligonucleotide-directed in vitro mutagenesis system from Amersham, UK.

2.3. *Assays for cyclin A and cyclin B degradation in extracts*

The translation mix containing [^{35}S]methionine-labelled cyclins was added to interphase or metaphase extracts in a ratio of 2 vols. to 30 vols. of extract. Recombinant starfish cyclin B was added to interphase extracts in a ratio of 7 vols. to 30 vols. of extract. After incubation at room temperature, 4 μl aliquots were transferred into 20 μl of SDS gel sample buffer.

2.4. *Immunoblotting*

For Western blotting, proteins were transferred from 10% polyacrylamide gels to nitrocellulose (Schleicher and Schuell) and reacted with rabbit antibodies specific for cyclin B or cdc2, followed by anti-rabbit IgGs conjugated to peroxidase (Amersham). Immunoblots were analysed using a chemiluminescent method (Amersham E.C.L.) according to the manufacturer's instruction. Cyclin B antiserum and affinity-purified antibodies were obtained as described by Lorca et al. [9]. cdc2 antibodies (NMPF) were generated against the N-terminal 12 amino acid peptide of p34^{cdc2}.

2.5. *H1 kinase assays*

To measure H1 histone kinase activities, the material retained on HSA beads was incubated for 10 min at room temperature in a reaction mixture containing 1 mg/ml H1 histones (Boehringer, Mannheim), 10 mM $MgCl_2$, 200 μM ATP (100 cpm/pmol) in 20 mM HEPES, pH 7.4. Reactions were stopped by adding 1 vol. of Laemmli buffer and the extent of histone phosphorylation was monitored by SDS-PAGE, followed by autoradiography.

3. RESULTS AND DISCUSSION

3.1. *Substitution of the invariant arginine residue in the destruction box of both cyclin A and B prevents their proteolysis in CSF extracts.*

In vertebrates, unfertilized eggs, although containing high cdc2 kinase activity, are prevented from exiting metaphase of meiosis II due to the presence of a cytostatic factor (CSF). Disappearance of CSF activity upon fertilization is caused by a transient increase in cytoplasmic free calcium. We have previously reported that cyclin proteolysis can be induced in extracts prepared from *Xenopus* eggs arrested at the second meiotic metaphase (CSF extracts) by activating a Ca^{2+}/calmodulin-dependent process which inactivates CSF [9]. *Patella* cyclin A and starfish cyclin B cDNA were mutated to convert the invariant arginine of the predicted destruction box (R41SALGTITN in *Patella* [13] or R31GALENISN in starfish [12]) respectively, to a cysteine or an alanine to create the derivatives, cyclin A-Cys41 and cyclin B-Ala31. These mutants and the wild-type cyclins, produced and labelled with [^{35}S]methionine in reticulocyte lysates, were added to CSF extracts. The degradation pathway was then turned on by adding 0.6 mM Ca^{2+}, which results in a transient increase of free Ca^{2+} concentration up to 0.1–1 μM [9]. As shown in Fig. 1, neither cyclin A-Cys41 nor cyclin B-Ala31 mutants were degraded under such conditions (Fig. 1, right). In

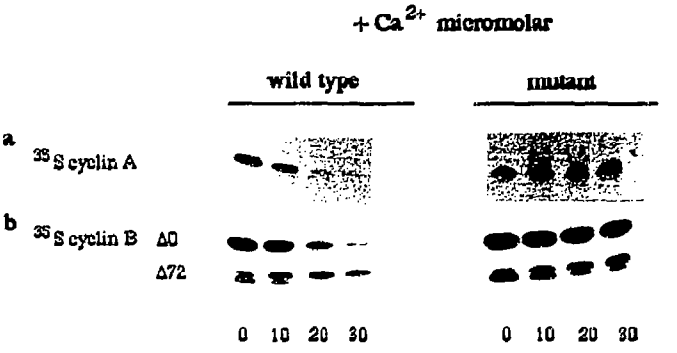

Fig. 1. An intact destruction box is required for cyclin A and cyclin B degradation after Ca^{2+} addition in CSF extracts. [^{35}S]methionine-labelled wild-type *Patella* cyclin A (a, left), cyclin A-Cys41 mutant (a, right), wild-type starfish cyclin B and a truncated form lacking 72 amino acids from the N-terminus (b, left) or cyclin B-Ala31 mutant (b, right) were added to CSF extracts. Samples (4 μl) were taken at the indicated times (min) after the addition of 0.6 mM $CaCl_2$ (final concentration) and analysed by SDS-PAGE and fluorography.

contrast, both wild-type cyclin A and B readily underwent degradation (Fig. 1, left). Besides the full-length cyclin B, in vitro translation of starfish cyclin B mRNA generates a protein lacking 72 amino acids from the N-terminus (Δ72), due to internal initiation at methionine 73 [14]. In contrast to the wild-type full-length cyclin, this truncated cyclin B did not undergo proteolysis, as a result of the truncation of the destruction motif, R31GALENISN (Fig. 1b).

3.2. *Wild type and mutant cyclins A and B degradation in interphase extracts*

In the next experiments, we used interphase extracts

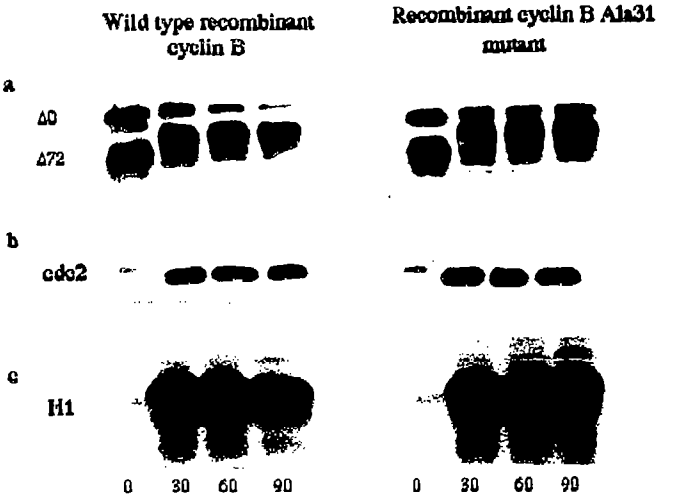

Fig. 2. Bacterially expressed wild-type cyclin B and cyclin B-Ala31 mutant can each induce histone H1 kinase activity when added to interphase extracts. (a) Recombinant starfish cyclin B (left) or cyclin B-Ala31 mutant (right) were added in interphase frog extracts. Samples were taken as a function of time and the materials purified on HSA beads were analysed either for cyclin B (a) or cdc2 (b) content by immunoblotting, or for histone H1 kinase activity (c).

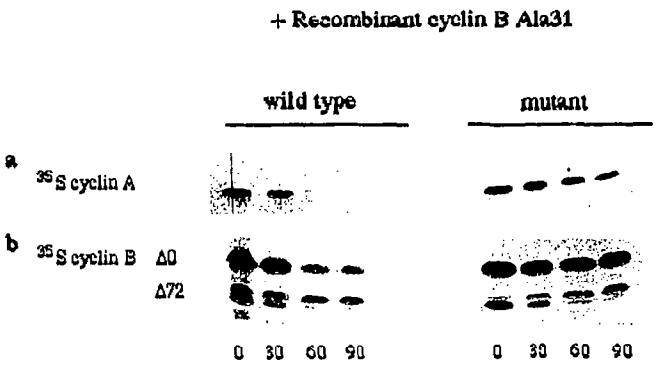

Fig. 3. Cyclin B-Ala31–cdc2 kinase switches on degradation of wild-type cyclin A and cyclin B but not that of cyclin A-Cys41 and cyclin B-Ala31 mutants. Recombinant starfish cyclin B-Ala31 was added to interphase *Xenopus* extracts simultaneously with [^{35}S]labelled wild-type *Patella* cyclin A (a, left), cyclin A-Cys41 mutant (a, right), wild-type starfish cyclin B (b,left) or cyclin B-Ala31 mutant (b, right). Samples were taken at the indicated time after addition of cyclins. [^{35}S]labelled cyclin destruction was monitored by SDS-PAGE followed by autoradiography.

prepared from *Xenopus* eggs 40 min after activation, which do not contain any CSF activity or any significant amount of cyclin B-cdc2 kinase activity. As expected, cyclin A and B, both produced in the reticulocyte system, remained stable in such extracts (data not shown). To turn on the cyclin degradation pathway, we generated cyclin B-cdc2 kinase activity by adding wild-type starfish cyclin B or mutant cyclin B-Ala31, both produced in bacteria as C-terminal fusion proteins containing the serum albumin-binding region of streptococcal protein-G (see section 2). These recombinant proteins formed complexes with cdc2 in extracts and, as a consequence, histone H1 kinase activity was efficiently produced in interphase extracts reaching a level similar to that found in CSF extracts. This activity could be recovered on HSA beads, a protein that avidly binds the serum albumin-binding domain of streptococcal protein-G (Fig. 2c). Similar amounts of cdc2 and cdc2-associated H1 kinase activity were bound to the beads in the presence of wild-type or mutant cyclin B, indicating that cdc2 binding to cyclin B and activity are not modified by conversion of Arg-31 to Alanine (Fig. 2b and c).

Table I

Effect of ATP or Mg^{2+} depletion on [^{35}S]labelled cyclin A and cyclin B degradation in interphase *Xenopus* extracts in which recombinant starfish cyclin B was added to induce cyclin proteolysis

Extract treatment	Cyclin A degradation	Cyclin B degradation
None	+	+
Hexokinase-glucose	−	−
EDTA (5 mM)	−	−
EGTA (5 mM)	+	+

Patella cyclin A and cyclin A-Cys41, or starfish cyclin B and cyclin B-Ala31, produced and labelled with [^{35}S]methionine in reticulocyte lysate, were then added to extracts containing recombinant cyclin B-Ala31-cdc2 kinase. As shown in Fig. 3 (left), degradation of wild-type tracer cyclins A and B readily occurred when H1 kinase activity increased. In contrast, both mutants, cyclin A-Cys41 and cyclin B-Ala31, were resistant to proteolysis (right). In these experiments, cdc2 kinase activity remained high even after cyclin degradation was turned on, because recombinant cyclin B-Ala31 (as well as the truncated form arising in the bacterial preparation and lacking the destruction box) failed to undergo degradation (Fig. 2a, right). The somewhat long lag phase (30–60 min) observed between the addition of recombinant cyclin B-Ala31 and the destruction of the tracer cyclin is at least partially accounted for by the time required to generate cdc2 kinase activity. Similar results were obtained when recombinant starfish cyclin B-Ala31 was substituted for the wild-type protein (not shown), although in that case the full-length recombinant cyclin B underwent proteolysis (Fig. 2a, left). Ubiquitin-dependent degradation of cyclins requires ATP-Mg^{2+} [15]. As expected, we found that degradation of [^{35}S]methionine-labelled wild-type cyclin A and B was completely inhibited when interphase extracts were initially depleted of endogenous ATP by adding hexokinase (0.2 mg/ml) and β-D(+)-glucose (15 mM). The addition of 5 mM EDTA, a magnesium chelator, also inhibited cyclin destruction, while 5 mM EGTA, a calcium chelator, had no such effect (Table I).

Taken together the above experiments show that stability of cyclin A mutated in the consensus sequence RXALXXI (the destruction box) is due to its inability to be proteolyzed by the ubiquitin pathway, as already reported for cyclin B.

Acknowledgements: We thank Simon Galas for help in preparing the manuscript. A.D. is a recipient of a fellowship from the MRC (Canada). T.S. is the recipient of a scholarship from La Ligue Nationale Contre Cancer.

REFERENCES

[1] Dorée, M. (1990) Curr. Opn. Cell Biol. 2, 269–273.
[2] Nurse, P. (1990) Nature 344, 503–508.
[3] Lorca, T., Labbé, J.C., Devault, A., Fesquet, D., Capony, J.P., Cavadore, J.C., Le Bouffant, F. and Dorée, M. (1992) EMBO J. (in press).
[4] Murray, A.W., Solomon, M.J. and Kirschner, M.W. (1989) Nature 339, 280–286.
[5] Ghiara, J.B., Richardson, H.E., Sugimoto, K., Heuze, M., Lew, D.J., Wittenberg, C. and Reeds, S. (1991) Cell 65, 163–174.
[6] Luca, F.C., Shibuya, E.K., Dohrmann, C.E. and Ruderman, J.V. (1991) EMBO J. 10, 4311–4320.
[7] Glotzer, M., Murray, A.W. and Kirschner, M.W. (1991) Nature 349, 132–138.
[8] Hershko, A., Ganoth, D., Pehrson, J., Palazzo, R.E. and Cohen, L.H. (1991) J. Biol. Chem. 266, 16376–16379.
[9] Lorca, T., Galas, S., Fesquet, D., Devault, A., Cavadore, J.C.

and Dorée, M. (1991) EMBO J. 10, 2087–2093.
[10] Gallant, P. and Nigg, E.A. (1992) J. Cell Biol. 117, 213–224.
[11] Félix, M.A., Pines, J., Hunt, T. and Karsenti, E. (1989) EMBO J. 8, 3059–3069.
[12] Labbé, J.C., Capony, J.P., Caput, D., Cavadore, J.C. Derancourt, J., Kaghad, M., Lelias, M., Picard, A. and Dorée, M. (1989) EMBO J. 8, 3053–3058.
[13] Van Loon, A.E., Colas, P., Goedemans, H.J., Néant, I., Dalbon, P. and Guerrier, P. (1991) EMBO J. 10, 3343–3349.
[14] Lorca, T., Fesquet, D., Zindy, F., Le Bouffant, F., Cerruti, M., Brechot, C., Devauchelle, G. and Dorée, M. (1991) Mol. Cell Biol. 11, 1171–1175.
[15] Luca, F.C. and Ruderman, J.V. (1989) J. Cell Biol. 109, 1895–1909.