Identification of a functional destruction box in the *Xenopus laevis* aurora-A kinase pEg2

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Received 13 September 2001; revised 12 October 2001; accepted 15 October 2001

First published online 26 October 2001

Edited by Giulio Superti-Furga

Abstract Like for all aurora-A kinases, the *Xenopus* pEg2 kinase level peaks in G_2/M and is hardly detectable in G_1 cells, suggesting that the protein is degraded upon exit from mitosis as reported for the human aurora-A kinase. We identified for the first time a sequence RxxL in the C-terminal end of the kinase catalytic domain. Mutation of this sequence RxxL to RxxI suppresses the ubiquitination of the protein as well as its degradation. The sequence RxxL corresponding to the pEg2 functional destruction box has been conserved throughout evolution in all aurora kinases including aurora-A, -B and -C. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Aurora-A; Proteasome; Degradation; *Xenopus*

1. Introduction

The mitotic aurora-A kinase is an oncogene, and overexpression detected in tumour cells is usually associated with the presence of the 20q13 amplicon which comprises the kinase gene [1,2]. However in few cases aurora-A overexpression is detected without 20q13 amplification indicating that other mechanisms might be involved in aurora-A overexpression. One of them could be an abnormal stability of the protein.

Many proteins involved in mitosis must be inactivated or degraded when the cell enters the following G_1 phase of the cell cycle. A dedicated degradation machinery known as the APC/C (anaphase promoting complex/cyclosome) identifies proteins previously ubiquitinated to be eliminated. Two major pathways have been identified so far depending on two different APC/C activators, Cdc20 and Cdh1 [3]. Although both pathways are active during somatic cell cycle, the Cdh1 pathway is absent during *Xenopus* early embryogenesis [4]. Proteins targeted by these degradation pathways must possess a destruction box recognised by the degradation machinery. Two different sequences have been described: a D-box and a KEN-box. The D-box contains two invariable conserved residues, an arginine and a lysine, separated by two amino acids [5]. The minimal required sequence RxxL is generally localised in the N-terminus domain of the protein [5,6]. The KEN-box which stands for a stretch of lysine/glutamic acid/asparagine residues could be functional in any part of the protein [7].

Human aurora-A has been found to be associated with the APC/C [8], but although the kinase is ubiquitinated in vivo and degraded through a mechanism sensitive to APC/C inhibitors, no functional destruction box has been identified yet [9,10]. We report here the identification of a functional degradation box in the *Xenopus* aurora-A kinase

2. Materials and methods

2.1. Reagents

Monoclonal antibodies 1C1 detect both the histidine-tagged and the endogenous pEg2 whereas 6E3 detects only the histidine-tagged pEg2 [11,12].

2.2. Cell culture

Xenopus XL2 cell extracts were prepared from G_1 synchronised cells as described by Bastians et al. [13,14]. The cells were harvested, incubated on ice for 20 min and lysed by sonification at 4°C and cellular debris was removed by centrifugation at $14\,000 \times g$ for 30 min. The cell lysate was then collected, aliquoted, and frozen in liquid nitrogen to be stored at −80°C.

2.3. Recombinant proteins

pEg2 coding sequence was inserted in the bacterial expression vector pET21 (Novagen) as previously described [11]. The cyclin-like destruction boxes were deleted by restriction enzyme digestions, and the deleted fragment was replaced by a short DNA fragment containing an internal *Bam*HI site to control the construction. This fragment was obtained by hybridisation of two oligonucleotides. Deletion of D_1-box was obtained by digestion of pET21-pEg2 by *Bsr*GI/*Eco*RI, deletion of D_2 by *Eco*RI/*Xho*I and deletion of D_1/D_2-boxes by *Bsr*GI/*Xho*I. D_1-box was replaced by 5′-GTACACCGGATCCG-3′ hybridised with 5′-AATTCGGATCCGGT-3′, D_2 by 5′-AATTCGGATCCC-3′ hybridised with 5′-TCGAGGGATCCG-3′, D_1/D_2-boxes by 5′-GTACACCGGTCCC-3′ hybridised with 5′-TCGAGGGACCGGT-3′. Deletion of the KEN-box in the N-terminal domain was obtained by PCR reaction on the pET21-pEg2 vector. The two primers used were 5′-AAG GCT AGC CAC AAG CCT TC-3′ containing a *Nhe*I site (underlined) and 5′-GGA GAT CTC GAG TTG GGC GGC TGG AAG GGG-3′ containing a *Xho*I site (underlined).

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0014-5793/01/$20.00 © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.
PII: S0014-5793(01)03048-4

The PCR product was directly cloned in pGEMT (Promega), and transferred in pET21 by double *Nhe*I/*Xho*I digestion/ligation. All histidine-tagged recombinant proteins were purified from BL21(DE3)-pLysS by Ni-NTA-agarose affinity chromatography following the manufacturer's instructions (Qiagen S.A.). All the purified proteins proved soluble and were conserved in 50% glycerol solution at −20°C.

2.4. Western blot analysis

After electrophoresis and transfer onto nitrocellulose membrane (Amersham Pharmacia Biotech) by standard methods, the membranes were blocked in TBST (20 mM Tris, pH 7.6, 137 mM NaCl, 0.1% Tween) containing 5% skimmed milk for 2 h at 4°C. The membranes were incubated with specific monoclonal antibodies for 1 h at 4°C in TBST containing 2.5% skimmed milk. After washing, the membranes were then incubated with peroxidase-conjugated secondary antibody (Sigma Chemicals). The immunocomplexes were revealed using peroxidase chemoluminescent substrates according to the manufacturer's instructions (NEN Life Sciences) and analysed on a FluoroImager (Molecular Dynamics).

2.5. In vitro degradation assay

2 μl of thawed cell extract was supplemented with an ATP regenerating system (1.5 mM ATP, 40 mM phospho-creatine, 80 μg/ml creatine kinase from Sigma Chemicals) and incubated with the recombinant purified protein in 7.4 mM HEPES pH 7.4, 5 mM KCl, 1.5 mM $MgCl_2$, 1 mM dithiothreitol. The amount of recombinant protein added to the extract never exceeded the amount of endogenous pEg2 to avoid any saturation of the degradation machinery. The reaction was incubated at 30°C and aliquots were taken at various time points (as indicated). Protein degradation products were separated by 12.5% SDS–polyacrylamide gel electrophoresis, transferred on nitrocellulose membrane, detected by Western blot using anti-pEg2 antibody and chemoluminescence and quantified using Image Quant software. Inhibition of the 26S proteasome was achieved by incubating the cell extract with 40 μM ALLN (acetyl-leucyl-leucyl-norleucinal) (Calbiochem, San Diego, CA, USA) prior to use in a degradation assay.

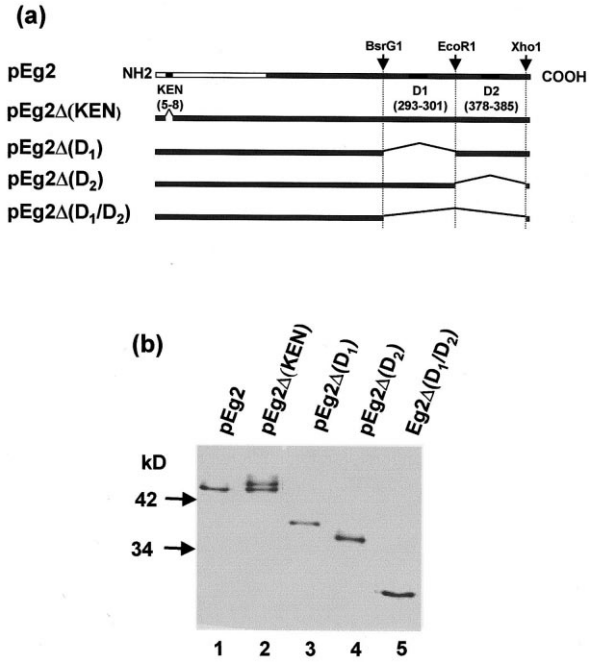

Fig. 1. Deletions of potential destruction boxes in pEg2. a: Schematic of recombinant pEg2 proteins used in the degradation assays (see Section 2). b: The quality of the preparation was controlled by Western blot using 1C1 monoclonal antibody (dilution 1:200). Lane 1: wild-type pEg2; lane 2: Δ(KEN); lane 3: Δ(D_1); lane 4: Δ(D_2); lane 5: Δ(D_1/D_2).

3. Results and discussion

We identified three potential destruction boxes in the *Xenopus* aurora-A kinase pEg2. Two RxxL D-boxes (D_1 and D_2) and one KEN-box. The D_1-box is located in the activation loop of the kinase catalytic domain and the xx residues in the sequence RxxL are two threonine residues, one of them being an activating threonine when phosphorylated [1]. The D_2-box and the KEN-box have potentially interesting positions because of their accessibility respectively in the C-terminal end (positions 6–8 in the wild-type protein) and the N-terminal end of the protein (positions 378–381 in the wild-type protein).

We have analysed the function of these three potential destruction boxes in an in vitro degradation assay [14] using recombinant histidine-tagged kinase pEg2 and various deleted versions of pEg2: Δ(D_1), Δ(D_2), Δ(D_1/D_2) and Δ(KEN) (Fig. 1a,b). Upon incubation in the extract 40% of the recombinant full length pEg2 was degraded within the first 30 min (Fig. 2a, first panel, and Fig. 2b). pEg2 was stabilised when the reaction was performed in the presence of the proteasome inhibitor ALLN (Fig. 2a, first and second panels). The D_1-box-deleted recombinant protein was also rapidly degraded, 90% after 30 min of incubation, and the degradation was also inhibited by ALLN (Fig. 2a, fourth and fifth panels). In contrast, 75% of the D_2-box-deleted protein remained after 120 min of incubation, indicating that the D_2-box was functional in our assay (Fig. 2a, sixth panel, and Fig. 2b). The deletion of the two boxes D_1 and D_2 completely stabilised the protein (Fig. 2a, last panel, and Fig. 2b). The KEN-box-deleted recombinant protein behaved just like the full length pEg2, indicating that this destruction box was not functional in our assay (Fig. 2a, third panel).

If one considers the aurora-A kinase family, the KEN-box is not present in the *Caenorhabditis elegans* aurora-A kinase sequences [16]. Additionally the second pEg2 cDNA version (*Xenopus laevis* is tetraploid) (GenBank accession number Z17207) does not contain any KEN-box. This should be a strong argument against a function of the KEN-box sequence in the degradation of the protein.

Giving the fact that the deletion of the D_2-box had the most dramatic effect on the stability of the protein we compared the C-terminal domain sequences of all aurora kinase catalytic domains (Fig. 3a). An alignment of the sequences revealed that a RxxL sequence is indeed found not only in every aurora-A sequence but in all the aurora kinases identified including aurora-B and aurora-C (Fig. 3a). The destruction box is invariably found in the C-terminal end of the catalytic domain of the kinase just downstream the kinase subdomain 11 (HxW) [17].

We then changed the leucine residue in the pEg2 D_2-box sequence RxxL by an isoleucine residue (Fig. 3b). A histidine-tagged recombinant protein carrying this point mutation was produced in *Escherichia coli*, purified and used in the degradation assay (Fig. 3c) and in a ubiquitination assay (Fig. 3d). Like the deletion of the D_2-box the point mutation L/I in the D_2-box stabilised the pEg2 kinase.

Two previous papers have reported the ubiquitination of the kinase [9,10]. Only the level of ubiquitination was reported to be destruction box dependent, without any correlation with the degradation of the protein. Giving the fact that aurora-A

has already been demonstrated to be ubiquitinated we analysed the effect of the D_2-box L/I point mutation in the capacity of pEg2 to be ubiquitinated. The ubiquitination reaction was performed with in vitro translated pEg2 in metaphase-arrested *Xenopus* egg extract in the presence of ubiquitin aldehyde which prevented hydrolysis of ubiquitin residues by isopeptidases. Although the presence of the CSF extract was not absolutely required for the ubiquitination its presence stimulated the reaction (compare Fig. 3d, lanes 1 and 4). Nevertheless pEg2 was not ubiquitinated when the incubation was carried out in the absence of ubiquitin aldehyde and methylated ubiquitin (Fig. 3d, lane 2) or in vitro mock translation (Fig. 3d, lane 3). In contrast, when ubiquitin was added readily to pEg2 incubated in the egg extract, ubiquitinated pEg2 conjugates were generated (Fig. 3d, lane 1). We did not detect any ubiquitination of the D_2-box L/I mutant indicating that the sequence is recognised by the ubiquitination machinery or that the ubiquitination occurs at a much lesser extent. The D_2 destruction box seems to target the ubiquitination of the protein as well as the degradation.

We demonstrate here in contradiction with what has been previously reported for the human aurora-A [9] that the *Xenopus* aurora-A contains a functional destruction box RxxL which sequence and position have been conserved from yeast to human. This sequence when deleted stabilised the *Xenopus* aurora-A protein. Another RxxL sequence (D_1) located in the activation loop of the kinase might be partially functional in our degradation assay. The *Xenopus* aurora-A kinase is totally insensitive to degradation when the two RxxL (D_1- and D_2-boxes) are deleted. There are at least three interpretations of this result. (i) The D_1-box sequence might also be an aurora-A functional destruction box which is unlikely to be the case because the sequence is not found in the mouse aurora-A kinase [16]. (ii) The D_1-box might become functional only when the D_2-box is deleted (e.g. in an artificial situation), 'mother nature always finds a way!'. (iii) In the *Xenopus*

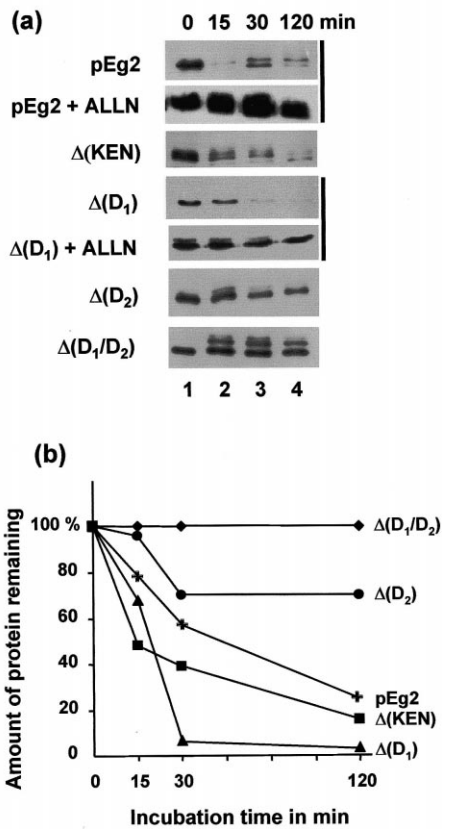

Fig. 2. pEg2 possesses a functional destruction box. Degradation kinetics of the various recombinant pEg2 proteins containing potential destruction box deletions were performed in XL2 cell extracts prepared from G_1 synchronised cells [13]. The reaction was incubated at 30°C. The specificity of the degradation was controlled by addition of the APC/C inhibitor ALLN. a: Reaction products were separated by 12.5% SDS–polyacrylamide gel electrophoresis and analysed by Western blot using the 1C1 monoclonal antibody (1:200). Aliquots of the reaction were taken at 0 (lane 1), 15 (lane 2), 30 (lane 3) and 120 min (lane 4). b: The Western blots were quantified with Image Quant and the amount of protein detected at various time points was expressed as a percentage of the protein detected at $t = 0$ min (input).

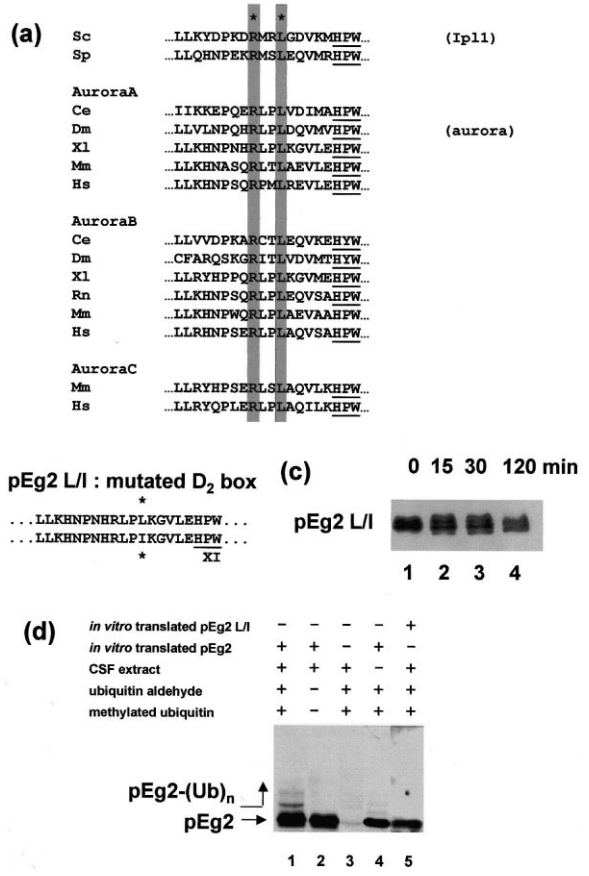

Fig. 3. Degradation and ubiquitination of L/I D_2-box-mutated pEg2. a: The sequence that corresponds to the pEg2 D_2-box was aligned with the D-box (RxxL) sequence of all aurora (-A, -B and -C) kinases from yeast to human. b: Point mutation of pEg2 D_2-box, the leucine residue of the sequence RxxL was changed to an isoleucine residue. c: Degradation kinetics of the L/I D_2-box-mutated pEg2. A point-mutated L/I D_2-box histidine-tagged recombinant pEg2 protein was prepared from *E. coli* and its stability tested in a degradation assay (same condition as in Fig. 2). d: In vitro translated pEg2 was incubated in the presence of metaphase-arrested *Xenopus* egg extracts [15] in the presence of ubiquitin aldehyde and methylated ubiquitin and immunodetected using the 6E3 monoclonal antibody [12] (lane 1). In lane 2, the reaction was carried out without ubiquitin aldehyde and methylated ubiquitin, and in lane 3 without in vitro translated pEg2. The ubiquitination of the point-mutated L/I D_2-box is shown in lane 5. The reaction mixtures were separated on a 12% SDS–polyacrylamide gel and transferred to nitrocellulose. pEg2 was immunodetected using the monoclonal antibody 1C1 (dilution 1:500).

aurora-A kinase this D_1-box sequence, RTTL, comprised the threonine that when phosphorylated activates the kinase [1]. A recent report has implicated a phosphorylation of the aurora-A kinase as a prerequisite to its degradation [10], a residue in this D_1-box might be involved in this mechanism. Also one can notice in our degradation assay that most of the recombinant proteins added to the extract shifted before degradation. This is particularly true for the D_1/D_2-boxes-deleted mutant (that lacks the activating threonine residue). Nevertheless a phosphorylation of the proteins seems to occur prior to degradation, the relationship between this phosphorylation and the degradation is currently being investigated. But the main point is that there is no doubt about the existence of a functional destruction box in the *Xenopus* aurora-A kinase.

The overexpression of the aurora-A kinases associated with centrosome amplification has been found in many tumours [1,2,18]. The main reason is the presence of the STK15 gene encoding aurora-A in the 20q13 amplicon that is at the origin of the overexpression of the kinase. But in some tumours although the amplicon is not present, an overactivity of the kinase is still associated with the cancer state of the cell [2]. It seems that in normal cell cycle, aurora-A has to be reset to coordinate nuclear cycle and centrosome cycle by the same mechanism, presumably at the restriction point in G_1 when the cell decides to duplicate both its DNA and centrosome [19–21]. Different hypotheses might explain the phenomenon, an over-transcription of the gene, an over-translation of the mRNA, an abnormal stability of the mRNA or an abnormal stability of the protein. In this last case the understanding of the mechanisms that control aurora-A degradation is of first importance. Those controls might not only concern aurora-A but also the other aurora kinases. Indeed aurora-A, aurora-B and aurora-C have been found overexpressed in cancer cells [22].

Acknowledgements: This research work was supported by the 'Centre National de la Recherche Scientifique', the 'Association pour la Recherche contre le Cancer', the 'Ligue Nationale contre le Cancer' and the 'Conseil Régional Bretagne'. A.K. was a recipient of the 'Fondation pour la Recherche Médicale', R.G. was a fellow of the French Government.

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