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The D-Box-activating domain (DAD) is a new proteolysis signal that stimulates the silent D-Box sequence of Aurora-A

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We have demonstrated previously that *Xenopus* Aurora-A is degraded at late mitosis by the APC/Fizzy-Related in a D-Box-dependent manner. Here we demonstrate that, although Aurora-B possesses the same D-Box as Aurora-A, Aurora-B is not degraded by this ubiquitin ligase. We have constructed a chimera Aurora-A/B with the N-terminus of Aurora-A and the C-terminus of Aurora-B and we have examined its degradation by APC/Fizzy-Related. We demonstrate that the N-terminus of Aurora-A confers degradation capacity on the C-terminus of Aurora-B and that this feature is blocked by mutation of the conserved D-Box sequence. We characterize the minimal degradation signal at the N-terminus of Aurora-A and demonstrate that its deletion blocks the degradation of this protein by APC/Fizzy-Related. Thus, we conclude that two different degradation signals are required for proteolysis of Aurora-A. The first one, which we designated D-Box-activating domain, within the N-terminal domain of Aurora-A confers the functionality to the second, a silent D-Box, present within the C-terminus of the kinase.

INTRODUCTION

The Aurora kinase family has been implicated in a wide variety of mitotic and meiotic processes including centrosome duplication, bipolar spindle formation, chromosome segregation and cytokinesis (Roghi *et al.*, 1998; Giet and Prigent, 2000; Giet *et al.*, 2002; Shannon and Salmon, 2002). It is difficult to subdivide this kinase family according to sequence homology, however it can be split into at least three different subfamilies on the basis of subcellular localization, namely Aurora-A, B and C (Giet and

Prigent, 1999; Nigg, 2001). Importantly, members of this family are overexpressed in a variety of cancers (Bischoff *et al.*, 1998; Zhou *et al.*, 1998), which underscores the pivotal role of the mechanisms which regulate Aurora kinase levels in the control of cell proliferation. In this regard, previous reports have demonstrated that Aurora-A is an unstable protein, the levels of which decrease through ubiquitin-dependent degradation in late mitosis-G₁ (Honda *et al.*, 2000; Castro *et al.*, 2002). As for Aurora-A, it has been reported that human Aurora-B and C activities also peak in mitosis (Bischoff *et al.*, 1998; Kimura *et al.*, 1999). However, little is known about the mechanisms that control the expression and the activity of these kinases during the cell cycle.

Events that occur during late mitosis are primarily governed by the ubiquitin-dependent degradation of key regulatory proteins. This ubiquitylation is mediated by the ubiquitin ligase, anaphase promoting complex (APC) (Zachariae and Nasmyth, 1999). Activation of the APC requires its association with WD-containing proteins. Two of these activators have been identified as Fizzy/Cdc20 and Fizzy-Related/Cdh1 (Sigrist and Lehner, 1997; Fang *et al.*, 1998; Lorca *et al.*, 1998; Kramer *et al.*, 2000). Whilst destruction of APC targets at the metaphase-anaphase transition is regulated by APC/Fizzy (Cohen-Fix *et al.*, 1996; Lorca *et al.*, 1998), APC-dependent degradation during late mitosis and early G₁ is controlled by Fizzy-Related (Visintin *et al.*, 1997; Pflieger and Kirschner, 2000). The switch from APC/Fizzy to APC/Fizzy-Related is induced by the inactivation of cdk1 which in turn, leads to destruction of Fizzy itself by the APC/Fizzy-Related complex (Visintin *et al.*, 1997; Shirayama *et al.*, 1998; Pflieger and Kirschner, 2000; Hagting *et al.*, 2002; Raff *et al.*, 2002). The

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Fizzy and Fizzy-Related forms of the APC present different substrate specificities. APC/Fizzy recognizes D-Box-containing proteins (Glotzer *et al.*, 1991; King *et al.*, 1996; Fang *et al.*, 1998), meanwhile APC/Fizzy-Related recognizes proteins containing either D-Box or KEN box sequences (Pfleger and Kirschner, 2000). We have previously demonstrated that Aurora-A kinase is degraded in late mitosis-G₁ by the APC/Fizzy-Related in a D-Box-dependent manner. Here, we further demonstrate the presence of a new complementary destruction signal, within the N-terminal domain of Aurora-A that we designed D-Box-activating domain (DAD), which is required to activate the D-Box-dependent proteolysis of this kinase by the APC/Fizzy-Related complex.

RESULTS AND DISCUSSION

Xenopus Aurora-B is not degraded by APC/Fizzy-Related during the cell cycle

Previous reports have demonstrated that, like Aurora-A (Kimura *et al.*, 1997; Bischoff *et al.*, 1998; Castro *et al.*, 2002), human Aurora-B protein levels increase at the G₂/M transition and then decrease at late mitosis (Bischoff *et al.*, 1998; Crosio *et al.*, 2002). Based on the sequence homology of these two kinases we were curious to determine whether the disappearance of Aurora-B was also mediated by APC-dependent ubiquitylation. We first asked whether Aurora-B might be degraded in an APC/Fizzy-dependent fashion. To address this issue, we developed an *in vitro* degradation assay, in which *Xenopus* metaphase II-arrested egg extracts (CSF extracts), previously supplemented with ³⁵S-radiolabelled *Xenopus* Aurora-B, or cyclin B as a control, were activated to exit meiosis by addition of calcium. We then measured Aurora-B levels by autoradiography at different times following addition of calcium. As expected, both Aurora-B and cyclin B2 were stable in the absence of calcium (Figure 1A, CSF-Ca²⁺). In contrast, the addition of Ca²⁺ did not trigger proteolysis of Aurora-B, whereas cyclin B2 was completely degraded (Figure 1A, CSF+Ca²⁺). To confirm these results, we examined the levels of endogenous Aurora-B and cyclin B2 by western blotting in a similar experiment. As shown in Figure 1B, endogenous cyclin B2 disappeared at 30 min following addition of Ca²⁺, whereas Aurora-B was stable throughout the entire time course. Thus, similarly to *Xenopus* Aurora-A, *Xenopus* Aurora-B is not a substrate of the APC/Fizzy complex.

We next investigated whether APC/Fizzy-Related was involved in the disappearance of Aurora-B at mitotic exit in somatic cells. To reconstitute a functional APC/Fizzy-Related complex, interphase *Xenopus* egg extracts were supplied with mRNA encoding this protein (Castro *et al.*, 2002). Both Aurora-A and B were stable in control interphase egg extracts that lack APC/Fizzy and APC/Fizzy-Related activities (Figure 1C, INT-Fzr). In contrast, as expected, Aurora-A was proteolysed within 60 min after addition to Fizzy-Related-containing extracts, whereas, Aurora-B was not (Figure 1C, INT+Fzr, upper and middle panel, respectively). Moreover, contrary to Aurora-A, endogenous Aurora-B was also stable throughout the entire experiment (Figure 1D). Thus, surprisingly, *Xenopus* Aurora-B does not seem to be an APC/Fizzy-Related substrate. According to our previous report on the D-Box-dependent degradation of Aurora-A by APC/Fizzy-Related and, because Aurora-B possesses the same

D-Box at the same position, we were expecting both kinases to be degraded by the same pathway. We then asked if *Xenopus* Aurora-B protein levels might remain constant throughout somatic cell cycle. To test this hypothesis, we examined the expression pattern of endogenous Aurora-B in *Xenopus* XL2 cells synchronized at different stages of the cell cycle. Contrary to Aurora-A whose levels clearly oscillate, we observed only a slight decrease of Aurora-B at the G₁ phase of the cell cycle. From these results we cannot exclude a partial degradation of this protein, however, our *in vitro* results show that, if this is the case, this proteolysis is not mediated by APC/Fizzy-Related. Thus, we concluded that, regulation of Aurora-B protein levels during the cell cycle is not mediated by an APC/Fizzy-Related proteolysis (Figure 1E).

The N-terminal domain of Aurora-A confers the degradation capacity on the C-terminal domain of Aurora-B

Despite the extent of sequence homology between Aurora-A and B kinases and the fact that Aurora-B contains the D-Box signal that induces proteolysis of Aurora-A (amino acids 331–334 of Aurora-B), Aurora-B is not degraded by APC/Fizzy-Related. Comparative analysis of the amino acid sequence of these two proteins shows that Aurora-A presents a longer N-terminal domain than Aurora-B, which shares homology. We hypothesized that in addition to the D-Box signal, proteolysis of Aurora-A is probably mediated by a supplementary degradation sequence situated within the N-terminal region of this kinase. To test this hypothesis we constructed a chimera of Aurora-A and Aurora-B in which the N-terminal domain of Aurora-A was merged with the C-terminal domain of Aurora-B (Figure 2A). Subsequently, we investigated whether this chimera was degraded upon the addition to Fizzy-Related-containing interphase extracts. As shown in Figure 2B, the chimerical form, Aurora-A/B, is indeed completely degraded in these extracts (upper panel), indicating that the N-terminal amino acid sequence of Aurora-A confers the degradation capacity on the C-terminus of Aurora-B.

In order to investigate if degradation of this chimera is regulated by the same mechanisms as Aurora-A kinase, we mutated the arginine and leucine residues of the D-Box motif of the chimera Aurora-A/B to alanine (R378A and L381A). Subsequently, we examined the proteolysis of chimerical mutated form upon addition to Fizzy-Related-containing extracts. The mutation of the D-Box strongly stabilized this protein (Figure 2B, lower panel), indicating that the D-Box sequence of Aurora-B is indeed activated by the presence of the N-terminal domain of Aurora-A.

Identification of the N-terminal sequence within the chimera Aurora-A/B required for its degradation

In order to identify the domain within the Aurora-A N-terminus involved in the activation of D-Box-dependent degradation of this kinase, a series of N-terminal deletions of the chimerical form of Aurora-A/B were constructed and the stability of each mutant was measured. These mutations are schematically represented in Figure 3.

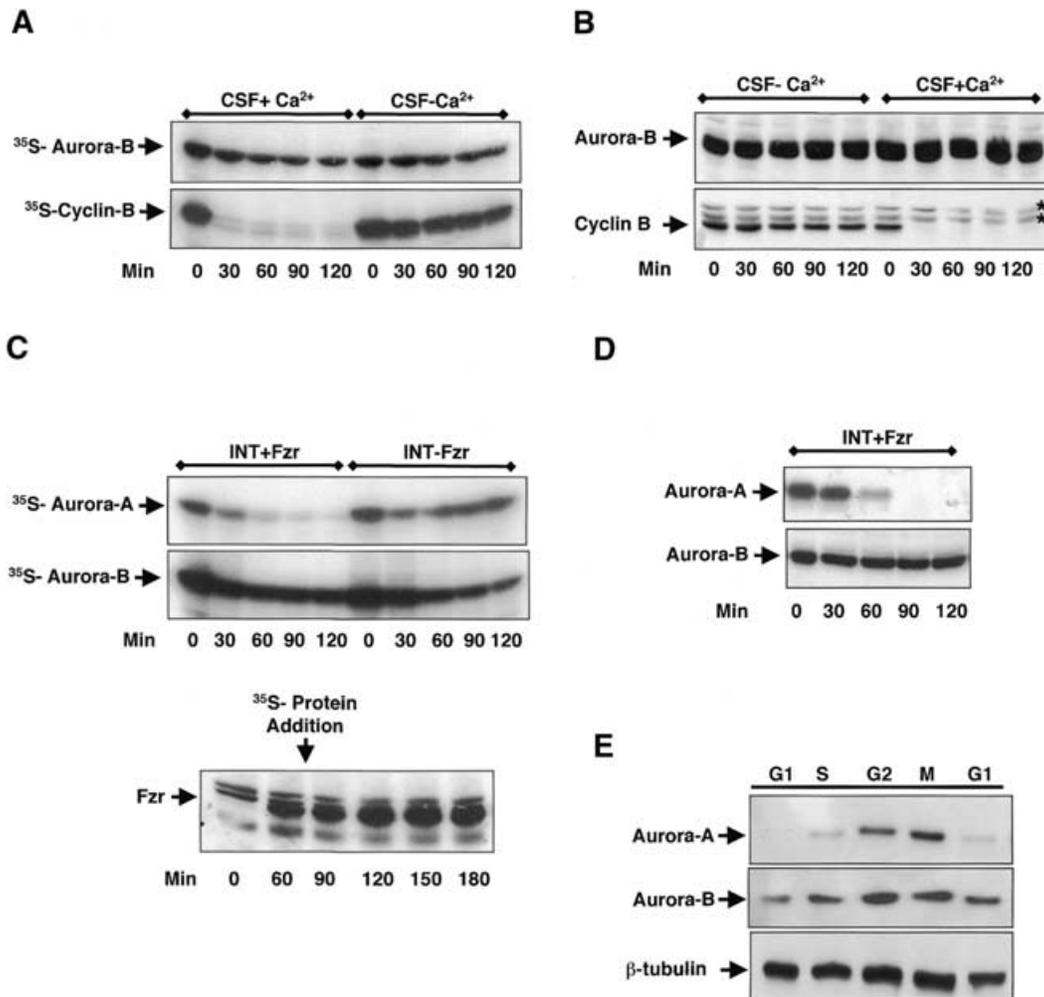


Fig. 1. *Xenopus* Aurora-B is not degraded by the APC/Fizzy-Related complex. (A) CSF extract (20 μ l) was supplemented with 2 μ l of *in vitro* translated 35 S-labelled Aurora-B and the same volume of 35 S-labelled cyclin B. Subsequently, a final concentration of 0.5 mM CaCl_2 was added where indicated (CSF+Ca $^{2+}$). Samples (2 μ l) were taken at different times and analysed by autoradiography. (B) Endogenous Aurora-B and cyclin B2 levels were analysed in a similar experiment by western blotting. * Represents unspecific bands recognized by the anti-cyclin B2 antibodies in the CSF extracts. (C) Fizzy-Related mRNA was added to interphase extracts (20 μ l) as indicated (INT+Fzr). One hour later 2 μ l of 35 S-labelled Aurora-A or the same amount of 35 S-labelled Aurora-B were added. Samples (2 μ l) were taken at different times and analysed by autoradiography. Fizzy-Related translation was verified by western blotting (lower panel, Fzr). The arrow at 60 min in the α -Fizzy-Related western blot indicates the time-point at which radiolabelled Aurora-A or B was added to the extract. (D) Interphase extracts (20 μ l) were supplemented with Fizzy-Related mRNA and endogenous protein levels of Aurora-A and Aurora-B were analysed by western blotting. (E) Cell cycle expression of *Xenopus* Aurora-A and Aurora-B. XL2 cells were synchronized at different phases of the cell cycle as described in Uzbekov *et al.* (1998). Protein levels were then analysed by western blotting with α -Aurora-A and α -Aurora-B antibodies (1/1000), and α - β -tubulin antibodies (1/1500).

The analysis of the N-terminal sequence of Aurora-A does not reveal any known degradation motif except for the presence of a putative KEN box in amino acids 6–8. To investigate a possible role of this putative motif in the chimera Aurora-A/B we analysed the degradation pattern of a Δ N(1–19) truncated form of this protein. According to our previous results in Aurora-A kinase (Castro *et al.*, 2002), the deletion of the first 19 amino acids which include the KEN box, did not confer stability to the chimerical form Aurora-A/B. Thus, the $^6\text{KEN}^8$ sequence is not functionally active in this chimera. To identify this new degradation signal, we continued our analysis by enlarging the N-terminal deleted zone of the chimera Aurora-A/B to 38 and 55 amino acids. Similarly to Δ N(1–19), mutant Δ N(1–38) of the chimera underwent normal degradation, whereas proteolysis

was blocked in mutant Δ N(1–55), revealing a potential degradation sequence at the N-terminal sequence of Aurora-A between amino acids 38 and 55.

Following our identification of this region as required for degradation of the Aurora-A/B chimera, we refined our search by creating three internal deleted forms of the chimera lacking amino acids 36–43, 44–50 and 51–55. The results of the degradation assays indicated that mutants Δ (44–50) and Δ (51–55) were clearly stable compared with mutant Δ (36–43), which was completely degraded. We then reduced the deletion sequence to four amino acids. We constructed mutants Δ (44–47), Δ (46–49), Δ (48–51) and Δ (52–55) of the chimera and observed that all of these mutated forms were stable in interphase extracts containing Fizzy-Related indicating that the new

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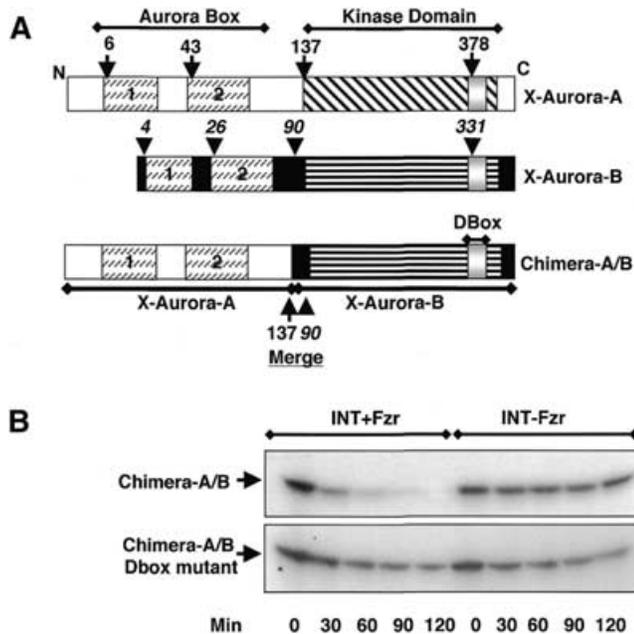


Fig. 2. The N-terminal domain of Aurora-A confers degradation capacity on the C-terminal domain of Aurora-B. (A) Schematic domain structure of Aurora-A, Aurora-B and Aurora-A/B chimera. (B) An interphase extract (20 μ l) was complemented (INT+Fzr) or not (INT-Fzr) with Fizzy-Related mRNA. One hour later both extracts were supplemented with 2 μ l of either *in vitro* translated 35 S-labelled A/B chimera or the D-Box mutated form of this chimerical protein. Samples of 2 μ l were then taken at different times and the two chimeras were analysed by autoradiography.

characterized N-terminal domain, that we here designate DAD, encompasses the sequence 44 VSAQRILGPSNV 55 of Aurora-A.

The DAD of Aurora-A is required for the D-Box-dependent proteolysis of this kinase

To investigate whether the DAD characterized in the artificially constructed Aurora-A/B chimera is also physiologically required for wild type Aurora-A to be degraded, we created a mutant of Aurora-A kinase in which this N-terminal sequence was deleted. Subsequently, we examined the degradation of this kinase in interphase extracts containing Fizzy-Related. As shown in Figure 4A, the kinase lacking amino acids 44–55 was clearly stable compared with the wild type form, confirming that this domain is indeed physiologically implicated in the regulation of D-Box-dependent proteolysis of Aurora-A.

We finally wanted to identify the minimal sequence of this N-terminal deleted region required for the degradation of Aurora-A. To answer this question we constructed a series of punctual mutated forms of Aurora-A in which every amino acid of the sequence 44 VSAQRILGPSNV 55 , except for alanine at position 46, was substituted by an alanine.

As shown in Figure 4B, mutants V44A, G51A and V55A were normally degraded in Fizzy-Related-complemented extracts indicating that these three amino acids are not required within the DAD to induce Aurora-A proteolysis. Moreover, proteolysis of S45A, Q47A and I49A mutated forms was slightly delayed whereas mutants, R48A, L50A, P52A, S53A and N54A of Aurora-A were clearly stabilized. Since proteolysis of S45A,

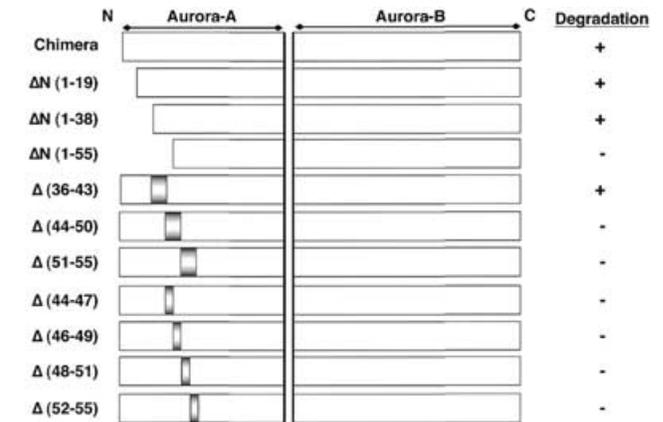


Fig. 3. Schematic representation of the sequence of Aurora-A/B chimera and the different mutants; N, N-terminus. Where internal sequences were deleted, they are depicted by shaded rectangles. The numbers in brackets indicate the deleted amino acids. All mutants of Aurora-A/B chimera were *in vitro* translated and incubated in Fizzy-Related-containing extracts to measure protein stability.

Q47A and I49A mutants were only partially affected, we deduced that they must not be essential for APC/Fizzy-Related degradation of Aurora-A. Thus we conclude that the minimal sequence of the DAD is 'RxLxPSN'.

Sequence alignment of this domain in *Xenopus*, mouse, rat and human indicated that this newly characterized domain is conserved during the evolution. As shown in Figure 4C, seven of the 12 amino acids of this domain are conserved in these species. Interestingly, these include amino acids R48, L50, P52, S53 and N54, which are all required for inducing *Xenopus* Aurora-A proteolysis, suggesting that DAD-dependent degradation of Aurora-A may be a general mechanism in vertebrates.

Thus, APC/Fizzy-Related-dependent degradation of Aurora-A is regulated by two degradation signals that act synergistically: the previously reported D-Box motif, and a new degradation domain with minimal sequence 'RxLxPSN', which confers its functionality to the D-Box motif and which probably accords Fizzy-Related specificity.

Conclusions

The presence of a D-Box degradation signal in the sequence of a considerable number of cell cycle substrates ensures the correct degradation of these proteins by the APC. The current data indicates that all substrates containing this degradation motif are proteolysed by both APC/Fizzy and APC/Fizzy-Related, except for Aurora-A kinase in which D-Box-dependent proteolysis is exclusively mediated by the APC/Fizzy-Related complex. This different behaviour can now be explained by our finding that degradation of Aurora-A is in fact mediated by two different signals: one within the N-terminus, that we term the D-Box-activating domain (DAD), and the other, the D-Box motif, within the C-terminus of its sequence. These findings agree with those of Littlepage and Ruderman (2002) that appeared when this manuscript was under submission. Based on our results obtained by comparison of Aurora-B and Aurora-A/B chimera, we postulate that the functionality of the D-Box sequence is in fact

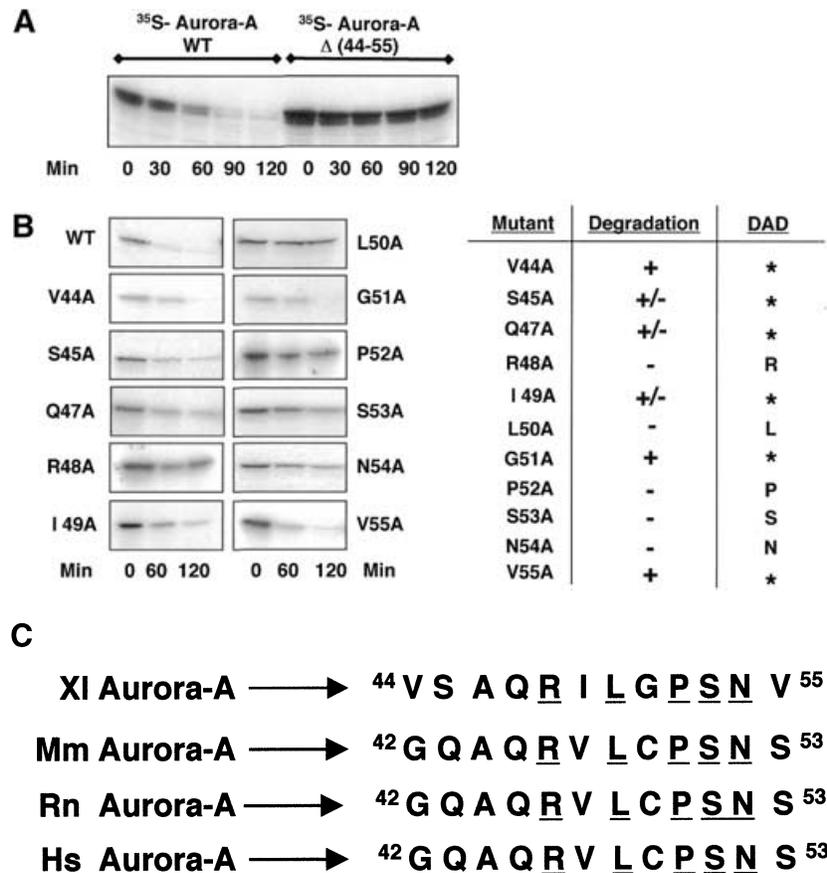


Fig. 4. The DAD is required for the physiological degradation of Aurora-A. (A) An interphase extract (20 μ l) was complemented with Fizzy-Related mRNA. One hour later the extract was supplemented with 2 μ l of either *in vitro* translated 35 S-labelled wild type Aurora-A or a mutated form of this kinase in which amino acids 44–55 were deleted. Samples of 2 μ l were then taken at different times and the two proteins were analysed by autoradiography. (B) Interphase extracts containing Fizzy-Related were supplemented with all the different radiolabelled punctual mutated forms of Aurora-A resulting from the substitution to alanine of every one of the amino acids from position 44–55. Samples of 2 μ l were then taken at 0, 60 and 120 min and Aurora-A protein levels were analysed by autoradiography. (C) Alignment of the DAD of *Xenopus* (XI), mouse (Mm), rat (Rn) and human (Hs) sequences of Aurora-A. Amino acids included in the DAD minimal sequence are underlined.

conferred by the presence of the DAD which may, in addition, accord APC/Fizzy-Related specificity. This is the first example described where a D-Box-dependent proteolytic event requires an additional motif located at some distance within the protein. In the current model a D-Box is defined as a degradation signal capable of conferring by itself an appropriate proteolysis of a non-degradable protein. Now, the present results open a new insight into the mechanisms controlling D-Box-dependent degradation by adding a new supplementary regulation level of the D-Box by other motifs. We do not know the mechanisms by which the DAD confers functionality to the D-Box sequence. One possibility would be that this motif appropriately localizes Aurora-A to different compartments within the cell, however, we observed a block of the degradation of the DAD mutated form of Aurora-A in *Xenopus* egg extracts where there is no compartmentalization. Another possibility would consider the three-dimensional structure of Aurora-A. The DAD could induce conformational changes to the protein and confer the structure required for the D-Box to be recognized by the APC/Fizzy-Related complex and, as a consequence, induce their association. Deletion of this motif would completely modify this three-dimensional conformation of Aurora-A and make the D-Box

sequence inaccessible by the APC/Fizzy-Related complex. Future studies will address this question.

METHODS

Immunization procedure, antibodies and immunofluorescence microscopy. A wild type *Xenopus* GST–Aurora-B fusion protein produced in *Escherichia coli* was used to immunize rabbits. Immune serum was affinity-purified on immobilized GST–Aurora-B. The *Xenopus* anti-Aurora-A, anti-cyclin B2 and anti-Fizzy-Related antibodies have been described elsewhere (Lorca *et al.*, 1998; Castro *et al.*, 2001). Immunofluorescence microscopy was developed as previously described (Castro *et al.*, 2002).

Translation and degradation in *Xenopus* egg extracts. Interphase and CSF extracts, as well as extracts competent in translation of *Xenopus* Fizzy-Related mRNA were prepared as previously described (Fesquet *et al.*, 1997). For protein degradation assays, 2 μ l of either 35 S-labelled cyclin B, Aurora-A, Aurora-B or chimera A/B and mutants were incubated at room temperature with 20 μ l of interphase extracts supplemented (1 h before) or not with Fizzy-Related mRNA.

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Site-directed mutagenesis. The chimeric form of Aurora-A and Aurora-B was obtained by overlap extension as previously described (Pogulis *et al.*, 1996). N-terminal mutations of the A/B chimera were obtained by PCR amplification. Internal or punctual mutations of Aurora-A and A/B chimera were developed by site-directed mutagenesis according to the manufacturer's recommendations (Stratagene). The sequences of the primers used to generate each mutant can be supplied on request.

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