Identification of XDRP1; a *Xenopus* protein related to yeast Dsk2p binds to the N-terminus of cyclin A and inhibits its degradation

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Using the N-terminus of cyclin A1 in a two-hybrid screen as a bait, we identified a *Xenopus* protein, XDRP1, that contains a ubiquitin-like domain in its N-terminus and shows significant homology in its C-terminal 50 residues to *Saccharomyces cerevisiae* Dsk2 and *Schizosaccharomyces pombe* dph1. XDRP1 is a nuclear phosphoprotein in *Xenopus* cells, and its phosphorylation is mediated by cyclin A-dependent kinase. XDRP1 binds to both embryonic and somatic forms of cyclin A (A1 and A2) in *Xenopus* cells, but not to B-type cyclins. The N-terminal ubiquitin-like domain of XDRP1, but not the C-terminal Dsk2-like domain, is required for interaction with cyclin A. XDRP1 requires residues 130–160 of cyclin A1 for efficient binding, which do not include the destruction box of cyclin A. The addition of bacterially expressed XDRP1 protein to frog egg extract inhibited the Ca²⁺-induced degradation of cyclin A, but not that of cyclin B. The injection of XDRP1 protein into fertilized *Xenopus* eggs blocked embryonic cell division.

Keywords: cyclin A/cyclin degradation/Dsk2-related protein/mitosis/ubiquitin-like protein

Introduction

Proteolysis plays an essential role in the periodic events of cell-cycle progression through the irreversibility caused by the degradation of cyclin and other proteins, such as Pds1/Cut2 (see review by King *et al.*, 1996a). Mitotic cyclins are rapidly degraded at the exit of mitosis and their degradation is necessary to enter the next cell cycle. The degradation of mitotic cyclins is mediated by the ubiquitin–proteasome machinery (Sudakin *et al.*, 1995; Hochstrasser, 1996; King *et al.*, 1996b), and this process requires an intact destruction box; a conserved nine-residue motif in their N-terminus (Glutzer *et al.*, 1991). Several other proteins involved in the regulation of mitotic events, such as chromosome segregation and spindle formation, possess a similar motif, and their degradation is also controlled by the ubiquitin–proteolytic machinery, activation of which is determined differently at least in part by members of the fizzy family of proteins including Cdc20/slp1 and Cdh1/Hct1 (Cohen-Fix *et al.*, 1996; Funabiki *et al.*, 1996; Juang *et al.*, 1997; Sgrist and Lehner,

1997). These proteins activate the selective ubiquitin ligase E3, known as the anaphase-promoting complex (APC) or cyclosome (King *et al.*, 1995; Sudakin *et al.*, 1995; Schwab *et al.*, 1997; Visintin *et al.*, 1997). The initiation of cyclin B degradation is also triggered by a direct cleavage of the N-terminal domain of cyclin by the 26S proteasome (Tokumoto *et al.*, 1997).

During the rapid cleavage cell cycles of early embryos, mitotic cyclins are destroyed only during a narrow time window (Hunt *et al.*, 1992; Sudakin *et al.*, 1995). Moreover, cyclins A and B are destroyed at slightly different times during mitosis (Lehner and O'Farrell, 1989; Minshull *et al.*, 1990; Pines and Hunter, 1990; Hunt *et al.*, 1992), with cyclin A being degraded prior to cyclin B, and the cyclin A-associated kinase activity increases and decreases earlier in the cell cycle than the cyclin B-associated kinase activity (Minshull *et al.*, 1990), suggesting that functional differences exist between cyclin A and cyclin B at distinct times of mitosis (Luca *et al.*, 1991).

Mitotic cyclins are characterized by two conserved motifs in the amino acid sequence; the destruction box and the cyclin box. The degradation of mitotic cyclins requires the destruction box of the N-terminus for its recognition by the ubiquitin-mediated proteolytic machinery (Glutzer *et al.*, 1991; Kobayashi *et al.*, 1992; Lorca *et al.*, 1992). Apart from the destruction box, the N-termini of mitotic cyclins contain other conserved motifs upstream of the start of the conserved cyclin box, which is required for association with Cdks. For example, the N-terminus of cyclin B1 contains a nuclear export signal LCQAFS, and both cyclins A and B contain autophosphorylation sites (Pines and Hunter, 1994; Klotzbucher *et al.*, 1996; Hagting *et al.*, 1998; Toyoshima *et al.*, 1998). Cyclin A contains a conserved motif, FxxVDE, between the destruction box and the start of cyclin box, which has recently been shown to be a cleavage site of ICE-like caspase for apoptotic proteolysis of cyclin (Stewart *et al.*, 1994; Stack and Newport, 1997). These imply that the conserved motifs in the N-terminus of mitotic cyclins between the stretch of the destruction box and the cyclin box could specify the distinct unknown functions of mitotic cyclins A and B during mitosis. To investigate the role(s) of these motifs with the aim of understanding the selective timing of degradation of cyclins A and B, we performed a yeast two-hybrid screen to isolate the cyclin A-specific interacting factors using the N-terminal domain of cyclin A as a bait. The major interacting species, XDRP1, was a *Xenopus* Dsk2-related protein in yeast that appears to be involved in the spindle checkpoint pathway. XDRP1 interacts with A-type cyclins, but not with cyclin B. Surprisingly, high levels of XDRP1 inhibited the degradation of cyclin A, but not that of cyclin B, and blocked cleavage cell division of embryos.

Fig. 1. Deduced amino acid sequence of *Xenopus* XDRP1. The deduced amino acid sequence of ORF of XDRP1 was shown, and its numbers are indicated at the right of the sequence. The N-terminal ubiquitin-like sequences (7–78) are shaded and seven identical residues with ubiquitin and four XDRP1-related proteins are shown by letters in a black rectangle. Similarly, the C-terminal yeast Dsk2p-like sequence (539–585) and identical residues are shown. Seven conserved repeats in the middle (171–240) and three conserved repeats (around 350–450) are underlined. The M-NPX sequences scattered are also shaded. The structures of frog XDRP1, budding yeast Dsk2, fission yeast dph1 and Nematoda F15C11.2 are schematically shown on the right of the sequence.

Results

Xenopus XDRP1 is a novel N-terminal ubiquitin-like protein

To search for factors that interacted with conserved domains in the N-terminus of cyclin A, we used the N-terminal 160 residues of *Xenopus* cyclin A1 fused to the Gal4 DNA-binding domain as a bait in a two-hybrid screen. Twenty out of 41 strongly interacting clones were found to be identical in sequence, containing a 2.3 kb insert of a full-length cDNA clone (Figure 1). The putative ORF of this clone, *Xenopus* Dsk2-related protein 1 (XDRP1), was composed of 585 residues with a calculated molecular mass of 62 kDa. A search of the databases revealed that the N-terminus of XDRP1 had strong homology to residues 39–70 of ubiquitin (53% identity), and that the C-terminus showed a high similarity to the C-terminus of budding yeast Dsk2 (Biggins *et al.*, 1996) and fission yeast dph1 (He *et al.*, 1998) (42 and 51% identity, respectively, in the 47 C-terminal residues), although the yeast proteins are smaller (39 and 36 kDa) (Figure 1). Nematode, mouse and human homologues were identified in the databases, although no full-length clones have yet been reported. The middle of the protein contains a number of short conserved repeated sequences, in which the sequence MXNPD/E/Q appeared, that were also observed in the yeast proteins (see Figure 1), and the sequences around residues 360 and 530 were unique to this protein. An alternative spliced form of XDRP1 consisting of 600 amino acid residues are isolated (data not shown), where 15 amino acid residues, EEIVIGET-ACVLEFK, are inserted into the ubiquitin-like domain, between amino acids 33 and 34 (Figure 1, indicated by a dot in the sequence).

XDRP1 persists in *Xenopus* cells as a nuclear phosphoprotein

We raised polyclonal antibodies against bacterially expressed XDRP1. Figure 2A shows that the antiserum against XDRP1 easily detected a single polypeptide with an apparent molecular weight of ~68 kDa by immunoblotting *Xenopus* egg extracts. The concentration of XDRP1 in eggs was estimated as 0.5–1 ng/egg, ~8–16 nM (data not shown), using immunoblotting with bacterially expressed GST–XDRP1 protein as a standard. XDRP1 protein was detected as a faster-migrating band through the early stages of oogenesis (Figure 2A). Two bands were detected in *Xenopus* embryos and cultured cells (lanes 3–5). The slower-migrating band detected in eggs (Figure 2B, lane 1) was converted to the faster-migrating one by treatment with lambda phosphatase (lane 3) and phosphatase inhibitors blocked this band shift (lane 4), from which we conclude that the upper XDRP1 band was a phosphorylated form of the lower one. A non- or hypo-phosphorylated form of XDRP1 was present in immature oocytes (Figure 2A, lane 1) and phosphorylated forms appeared during oocyte maturation (lane 2). At later stages of development and in cultured cells, both forms were detected (lanes 3–5). Dephosphorylated form appeared at 40–50 min after activating egg to drive the cell cycle (Figure 2C), although no typical cell-cycle pattern of phosphorylation was observed at its protein level. When ³⁵S-labelled XDRP1 in egg extracts was immunoprecipitated with anti-XDRP1 antibody, however, phosphorylated form was detected mainly at CSF-arrested extracts and non-phosphorylated form was detected in interphase (Figure 2D). Therefore, XDRP1 is phosphorylated at M phase, but not in interphase.

Fig. 2. XDRP1 is a phosphoprotein. (A) Immunoblotting of XDRP1 during *Xenopus* development. *Xenopus* oocytes, eggs, embryos (cleavage and blastula) and kidney culture cells (WAK) were taken and the samples were immunoblotted with anti-XDRP1 antibody. (B) XDRP1 is phosphorylated. XDRP1 was detected by immunoblotting with anti-XDRP1 antibody. Lane 1, CSF-arrested extracts; lane 2, WAK cells; lane 3, CSF-arrested extracts treated with λ phosphatase; lane 4, CSF-arrested extracts with λ phosphatase and phosphatase inhibitors; lane 5, CSF-arrested extracts diluted 7-fold with the buffer. (C) XDRP1 abundance during the cell cycle. *Xenopus* eggs activated by calcium ionophore were sampled at the times indicated. XDRP1 was detected by immunoblotting with anti-XDRP1 antibody. The M phase is indicated as an asterisk (*) at the bottom, where the H1 kinase activity is high (data not shown). (D) Phosphorylation of XDRP1 at M phase. *Xenopus* CSF-arrested extracts or interphase extracts were labelled for 60 min with 1 μ Ci/ μ l of [35 S]methionine in the presence of cyclin A1 mRNA. 35 S-labelled XDRP1 was immunoprecipitated from *Xenopus* egg extracts with anti-XDRP1 antibody, and analyzed by SDS-PAGE and autoradiography. M, *Xenopus* CSF-arrested extracts (lanes 1–2); and Int, interphase extracts (lanes 3–4). PI, preimmune serum (lanes 1 and 3); and I, immune serum (lanes 2 and 4). Control, the precipitates with anti-cyclin A1 antibody K3 (lane 5). Note a weak band in lane 2 (arrowhead).

To investigate the subcellular localization of XDRP1, cultured *Xenopus* cells were stained using anti-XDRP1 antiserum. XDRP1 protein was predominantly localized to the nucleus in interphase cells (Figure 3A), while in mitotic cells, no particular subcellular location was observed. XDRP1 was detected in both nuclear and cytoplasmic fractions of cell lysates (Figure 3B). The nuclear form had a lower mobility than the cytoplasmic one (lane 1), thus suggesting that the phosphorylated form of XDRP1 is located in the nucleus.

XDRP1 binds to full-length cyclin A1 and A2, but not to B-type cyclins

We tested binding of XDRP1 with cyclin A by growth and β -galactosidase in the yeast two-hybrid system, since

Fig. 3. XDRP1 locates at the nucleus. (A) Immunofluorescence of XDRP1. Asynchronous *Xenopus* cultured WAK cells were immunostained with anti-XDRP1 antibody (left columns). The same field was observed by counterstaining with DAPI for nuclear DNA (middle columns) and by a microscope to demonstrate the cell morphology (right columns). The asterisk indicates a mitotic cell. Bar, 20 μ m. (B) Subcellular fractionation. WAK cells were fractionated into the cytoplasmic and the nuclear fractions as described in the Materials and methods. Three micrograms of total proteins in each fraction were applied on the gel and were co-immunoblotted with both anti-XDRP1 antibody and anti-RCC1 antibody. Nuclear protein RCC1 was used as a control to check the fractionation. Lane 1, nuclear fraction; lane 2, cytosol; lane 3, total cell fraction.

XDRP1 was originally isolated by yeast two-hybrid screen using the N-terminal domain of cyclin A1 as bait (positions 1–160). Both assays showed XDRP1 to bind to the N-terminal domain of cyclin A1 (Figure 4A).

Next we tested the binding of cyclin A1 to XDRP1 in *Xenopus* egg extracts. The full-length cyclin A1 mRNA was translated in the presence of [35 S]methionine in frog egg extracts, as described in Materials and methods. Recombinant GST–XDRP1 was then added, followed by affinity chromatography on GSH–Sepharose to isolate the proteins that bound to the bacterially expressed GST–XDRP1. Figure 4B shows cyclin A1 was retained when GST–XDRP1 was added (Figure 4B, lane 6), and not with GST alone (lane 2). The binding of cyclin A to GST–XDRP1 was significantly lower than the binding of cyclin A to GST–CDK2, which acted as a positive control in this experiment (Figure 4B, lane 4). GST–XDRP1 also bound to *Xenopus* and human cyclin A2, the form of cyclin A found in somatic cells (Figure 4C, lanes 5 and 6). We also checked the binding of XDRP1 to cyclin A1 in yeast cells, where a full-length *Xenopus* cyclin A1

Fig. 4. XDRP1 binds to cyclin A, but not to cyclin B. (A) Binding of XDRP1 to cyclin A1 in yeast two-hybrid system. The N-terminal domain of *Xenopus* cyclin A1 (1–160) cloned into PAS404 was expressed in yeast and their histidine-prototrophic growth (left) and increased β-galactosidase activity (right) was tested. (B) Binding of XDRP1 to cyclin A1 in *Xenopus* extracts *in vitro*. ³⁵S-labelled *Xenopus* cyclin A1 was precipitated with GST (lanes 1 and 2), *Xenopus* GST-CDK2 (lanes 3 and 4) and GST-XDRP1 (lanes 5 and 6) in the CSF/Retic-mixed extracts, followed by SDS-PAGE and autoradiography. Top panel, translation; bottom panel, GST-bound cyclin A1. (C) Binding of XDRP1 to other A-type cyclins. *Xenopus* cyclins A1 and A2 and human cyclin A2 were ³⁵S-labelled in the extracts and their binding abilities to XDRP1 were tested by GST-XDRP1 pull-down. Exposure time, overnight. Translation (top panel) and GST-XDRP1 precipitates (bottom panel). (D) Binding of XDRP1 to cyclin A2 in transfected cells. T7-His₆-tagged XDRP1 were precipitated from the WAK cell extracts as described in the Materials and methods. The precipitates were immunoblotted with either anti-T7 antibody (lanes 5–8, top panel) or anti-HA antibody (lanes 5–8, bottom panel). Expression was checked by immunoblotting of the extracts (15–30 μl) with anti-T7 antibody for XDRP1 (lanes 1–4, top) or anti-HA antibody for cyclin A2 (lanes 1–4, bottom). (E) Binding of XDRP1 to cyclin A, but not cyclin B. *Xenopus* cyclin A1, cyclin B1 and cyclin B2 mRNAs were translated in the CSF/Retic-mixed extracts and assayed for XDRP1 binding. Left panel, translation; right panel, GST-Sepharose beads bound. Exposure time, 2 days.

overproduced from a yeast-inducible vector could be isolated by addition of GST-XDRP1 to the lysate followed by affinity chromatography on GSH-Sepharose and immunoblotting with anti-cyclin A1 antibody (data not shown).

Next we tested interactions between XDRP1 and cyclin A in *Xenopus* cells. Constructs of T7-His-tagged XDRP1 and HA-tagged cyclin A2 were transfected into WAK cells and both proteins were expressed *in vivo* (Figure 4D, lane 4). Then, T7-His-XDRP1 proteins were precipitated with TALON metal affinity resin, and XDRP1-bound materials were immunoblotted with anti-HA antibody against HA-cyclin A2. Figure 4D shows that XDRP1 interacted with cyclin A2 in cells (lane 8). When immunoprecipitation using anti-XDRP1 antibody was carried out after translating cyclin A1 mRNA in the *Xenopus* egg extracts, labelled cyclin A1 was weakly co-precipitated with anti-XDRP1 antibody (see arrowhead in Figure 2D). It proved difficult to demonstrate interactions between them in *Xenopus* cell extracts using immunoprecipitation with either anti-XDRP1 or anti-cyclin A1 antibodies, followed by immunoblotting. It was clear that the interaction between XDRP1 and cyclin A was weaker than that between CDK2 and cyclin A (see Figure 4B).

Next, to test whether XDRP1 bound to B-type cyclins,

cyclins A and B were labelled with [³⁵S]methionine in the egg extracts and tested for their ability to bind to GST-XDRP1. XDRP1 bound to *Xenopus* cyclin A1 (Figure 4E, lane 5) in the frog egg extract, but not to cyclin B1 (lane 6) or cyclin B2 (lane 7). Even when both cyclins A and B were co-translated, neither cyclin B1 nor cyclin B2 bound to GST-XDRP1 (Figure 4E, lanes 8 and 9). Therefore, XDRP1 appears to bind to cyclin A specifically and not cyclin B. The same results were obtained by two-hybrid assay (both histidine-prototrophic growth and increased β-galactosidase activity) using the equivalent peptides of the N-terminal cyclin B (positions 1–132 in cyclin B1 and 1–127 in cyclin B2) (data not shown).

XDRP1 binds to the cyclin A-CDK complex and XDRP1 phosphorylation depends upon cyclin A-dependent kinase

To test whether XDRP1 could bind to cyclin A-CDK complexes, Cdc2 was labelled with [³⁵S]methionine in egg extracts, and affinity chromatography with GST-XDRP1 was performed as before. Both cyclin A1 and Cdc2 were retained on GSH-Sepharose in the presence of GST-XDRP1 (Figure 5A, lane 6). A similar result was obtained for CDK2 (data not shown). To confirm that XDRP1 actually binds to the cyclin-CDK complex, we

Fig. 5. XDRP1 is phosphorylated by cyclin A-dependent kinase. **(A)** Co-precipitation of cyclin A1 and Cdc2 with XDRP1. Cyclin A1 and Cdc2 mRNAs were co-translated in CSF/Retic-mixed extracts with [35 S]methionine, and the precipitates with GST-XDRP1 were subjected to autoradiography. **(B)** H1 kinase activity of the GST-XDRP1 precipitates. Cyclin A1 mRNA was translated in CSF/Retic extracts and the precipitates with GST-XDRP1 was assayed for histone H1 kinase activity. Expression of cyclin A1 (top panel), cyclin A1 bound to GST-XDRP1 (middle panel) and histone H1 kinase activity (bottom panel). **(C)** Phosphorylation of XDRP1 depends on cyclin A. GST-XDRP1 was incubated in cyclin A1-expressed CSF/Retic-mixed extracts and detected by immunoblotting with anti-XDRP1 antibody as described in the Materials and methods (top panel). The expression of cyclin A1 were checked by immunoblotting with anti-cyclin A1 antibody (bottom panel). **(D)** Phosphorylation of XDRP1 is blocked by butyrolactone I. GST-XDRP1 was incubated with different concentrations of butyrolactone I; 0 nM (lane 2), 50 nM (lane 3) and 200 nM (lane 4). GST-XDRP1 in the absence of cyclin A1 (lane 1). For details see Materials and methods.

examined whether the GST-XDRP1 displayed histone H1 kinase activity. Figure 5B shows that the GSH-Sepharose beads carrying GST-XDRP1 possessed H1 kinase activity (Figure 5B, lane 4) only when cyclin A1 mRNA was translated in the extract (lanes 3 and 4). GST alone did not retain cyclin A or significant kinase activity (lanes 1 and 2).

Since XDRP1 binds to the cyclin A-CDK complex, whether XDRP1 phosphorylation is mediated by cyclin A-dependent kinase or not was tested (Figure 5C). GST-XDRP1 was phosphorylated when cyclin A1 was translated in egg extracts (Figure 5C, lane 2), where CDK activity was elevated by cyclin A1 expression (data not shown). Increasing amounts of butyrolactone I added to the extracts inhibited phosphorylation of GST-XDRP1 (Figure 5D, lanes 3 and 4). GST-XDRP1 was directly phosphorylated by the immuno-purified cyclin A-cdc2 complex *in vitro* (data not shown). Therefore, cyclin A-dependent kinase contributes to phosphorylation of XDRP1.

The ubiquitin-like domain of XDRP1 is necessary but not sufficient for interaction with cyclin A

We tested which domains of XDRP1 were required for the interaction with cyclin A. N- and C-terminal deletions were made and their binding abilities for cyclin A1 were tested by two-hybrid assay (Figure 6A). Deletions of the ubiquitin-like domain in the N-terminus (76–585) abolished its binding with cyclin A1, but that of the C-terminal conserved Dsk2-like domain did not (1–538), showing a requirement for the N-terminal ubiquitin-like domain for XDRP1 to interact with cyclin A. The non-conserved middle region of XDRP1 was also required for

its binding to cyclin A1, however, because the N-terminus alone showed no interaction.

Residues surrounding the major phosphorylation site in cyclin A are necessary for interaction with XDRP1

To define the region of cyclin A1 that was required for XDRP1 binding, we prepared a number of mutants and synthesized the corresponding 35 S-labelled proteins in egg extracts (Figure 6B, upper panel) followed by affinity chromatography using GST-XDRP1 (Figure 6B, lower panel). The N-terminal fragment (1–160), which was originally used as a bait for two-hybrid screening, bound to XDRP1 as expected, but its binding was rather poor in comparison with full-length cyclin A1 (Figure 6B, lanes 2 and 3). No signal was detected with GST alone (lane 1). The construct comprising residues 133–418 of cyclin A1 bound to XDRP1 as well as the full-length protein (lanes 3 and 7), whereas residues 161–418 did not bind at all (lane 8). The construct with an internal deletion, Δ 88–144, bound to XDRP1 with slightly reduced efficiency (lane 5) compared with full-length cyclin A1 (lane 3). These results suggest that the residues between 133 and 160 of cyclin A1 are required for XDRP1 binding. A destruction-box mutant ATVA bound to XDRP1 normally in egg extracts (lane 4). Therefore, the destruction box, which is necessary for the ubiquitin-mediated proteolysis of mitotic cyclins, was not required for XDRP1 binding. The sequence around 133–160 contains a motif conserved among A-type cyclins, in which phosphorylation site SP at 136/137 surrounded by other partially conserved residues around 130–145 is reasonably well conserved (Figure 6C).

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Fig. 7. GST-XDRP1 selectively inhibits degradation of cyclin A, and blocked the cleavage cell division in *Xenopus* embryo. **(A)** Selective inhibition of degradation of cyclin A, but not that of cyclin B. Degradation assays were carried out in *Xenopus* CSF-arrested extracts as described in Materials and methods. Cyclin degradation was induced by adding 0.4 mM calcium to the extracts. In this experiment, 100 ng/ μ l of purified GST-XDRP1 was added to the extracts. The addition of GST alone (100 ng/ μ l) had no inhibitory effect on cyclin destruction. Panel a, XB buffer-added extracts; panel b, GST-XDRP1-added extracts; panel c, XDRP1-depleted extracts. **(B)** Microinjected GST-XDRP1 blocked embryonic cell division in *Xenopus*. GST-XDRP1 (60 ng/embryo) was injected into the fertilized eggs as described in Materials and methods. GST was injected as a control (60 ng/embryo). The eggs injected the lower concentration of GST-XDRP1 (30 ng/embryo) cleaved slowly, and as a result, an injected embryo induced incomplete gastrulation (data not shown).

***Xenopus* XDRP1 contains a ubiquitin-like domain in the N-terminus and shares the C-terminal conserved domains with yeast Dsk2**

XDRP1 contained a short ubiquitin-like sequence in its N-terminal end (53% identity to the C-terminal half of ubiquitin). A family of proteins containing such a ubiquitin-like domain in its N-terminus has been reported; yeast Dsk2 (Biggins *et al.*, 1996) and dph1 (He *et al.*, 1998), Rad23 (Watkins *et al.*, 1993) and *Xenopus* An1a/b (Linnen *et al.*, 1993). A similarity was also observed in the C-termini of *Xenopus* XDRP1 and yeast Dsk2 (42% identity), and in nine repeats with nine or 10 amino acid residues in the middle region of both peptides (see Figure 1, positions 170–240 and 370–440). Based on these molecular features, XDRP1 and Dsk2 probably share the same function in the cell cycle, which may be related somehow to the cell cycle. DSK2-1 was originally isolated as a suppressor of a *kar1* allele defective for SPB duplication in budding yeast, and DSK2 overexpression caused mitotic defect with short spindle (Biggins *et al.*, 1996). Therefore, it is possible that XDRP1 may be involved in the spindle function in mitosis, perhaps through the interaction with cyclin A. The involvement of the cyclin A function in microtubule nucleation in mitotic spindle formation has been noted previously by several investigators (Buendia *et al.*, 1992; Maldonado-Codina and Glover, 1992; Verde *et al.*, 1992), although the function *in vivo* of cyclin A is still unclear. The recently identified *Schizo-*

saccharomyces pombe dph1⁺, a homologue of DSK2, causes metaphase arrest with a short mitotic spindle when overexpressed (He *et al.*, 1998), which is reminiscent of our finding that high levels of GST-XDRP1 can inhibit cyclin A proteolysis and block cell division (Figure 7).

A new motif in the N-terminal domain of cyclin A is required for XDRP1 via its N-terminal ubiquitin-like domain

Two conserved motifs in the N-terminal domain of cyclin A have been characterized so far; the destruction box and the FxxVDE motif (Stewart *et al.*, 1994). The destruction box is required for the recognition by the ubiquitin-mediated APC-dependent pathway (Glotzer *et al.*, 1991). The conserved FxxVDE motif has recently been reported as a cleavage site for ICE-like caspase in apoptotic proteolysis (Stack and Newport, 1997). Neither of these motifs was required for XDRP1 binding (Figure 6). Rather, XDRP1 interacted with residues 133–160, downstream of the FxxVDE motif. This region contains a phosphorylation site conserved in A-type cyclins, -139SPxMxS144-, and acidic residues around position 150 (see Figure 6C). The interaction of XDRP1 with this new motif might be involved in the regulation of cyclin degradation as discussed below, while the N-terminal ubiquitin-like domain of XDRP1 is found to be required for cyclin A binding. We note that XDRP1 is a stable protein and there was no evidence that its N-terminal ubiquitin-like sequence was processed from full-length XDRP1.

XDRP1 is a candidate substrate for cyclin A-dependent kinase

Our results indicate that XDRP1 might be a candidate substrate for the cyclin A-CDK. XDRP1 is sharply phosphorylated during oocyte maturation (Figure 2A), and the activity of XDRP1 phosphorylation is shown to be present at M phase in egg extracts (Figure 2D). Although we have shown previously that the level of cyclin A1 proteins is low in *Xenopus* eggs and cyclin A-cdc2 kinase fails to be detected in matured egg extracts (Kobayashi *et al.*, 1991), the cyclin A-dependent kinase, even though its activity is low, would contribute to XDRP1 phosphorylation. Besides being phosphorylated by cyclin A-dependent kinase itself, XDRP1 would be phosphorylated by either other kinases or the kinases activated by cyclin A. However, cyclin B-dependent kinase (MPF) would not contribute to XDRP1 phosphorylation, because cyclin B does not bind to XDRP1 (Figure 4E) and no phosphorylated form of GST-XDRP1 was observed when cyclin B was expressed in the extracts (unpublished data).

The binding of XDRP1 with cyclin A does not require a state of XDRP1 phosphorylation, because cyclin A1 can bind to both phosphorylated and dephosphorylated forms of XDRP1 (data not shown). But, vice versa, phosphorylation of XDRP1 depends upon its binding to cyclin A, because the N-terminal deletion of cyclin A (161–418) failed to phosphorylate XDRP1 completely (data not shown). Taken together, XDRP1 would be associated with the cyclin A-CDK complex first (Figure 5B), and then phosphorylated by cyclin A-dependent kinase at M phase.

The dominant fraction of XDRP1 is located at the nucleus as the phosphorylated form (Figure 3A and B). Cyclin A is known to be located predominantly at the

nucleus throughout the cell cycle (Pines and Hunter, 1991), and its sequence does not contain a distinct nuclear localization signal. The phosphorylation of XDRP1 may be involved in localization of cyclin A at the nucleus. XDRP1 contains six putative SP sites in the middle stretch of its sequence. We are currently working on the mutation analysis of XDRP1 phosphorylation.

Possible role of XDRP1 in the regulation of cyclin A degradation in mitosis

XDRP1 binds to a conserved motif in the N-terminus of cyclin A *in vitro* and selectively inhibits cyclin A degradation in egg extracts (Figures 6 and 7A). Because the N-terminus of mitotic cyclin including the destruction box is required for ubiquitin-dependent proteolysis, the binding of XDRP1 to the N-terminus of cyclin A may interfere with the interaction and/or the access of some protein(s) to cyclin A, which is required for proteolytic machinery. This would be the reason why XDRP1 selectively inhibits cyclin A degradation in *Xenopus* egg extracts and embryos.

The state of XDRP1 phosphorylation is not directly related to the binding ability with cyclin A or cyclin A-CDK (data not shown). Rather, the conformational change of cyclin A-CDK complex through XDRP1 binding to cyclin A would be an important step for cyclin A degradation, probably by protecting the cyclin substrate from the attack of the ubiquitin-dependent proteasome pathway. So far, we cannot detect a strong interaction between XDRP1 and cyclin A *in vivo*. However, we suspect that XDRP1 might function through interaction with cyclin A in cells, because XDRP1 is specific for cyclin A as characterized by their interaction in cells and by a block of its degradation. A simple idea to explain our results is therefore that the selective binding of XDRP1 with cyclin A may be involved in the negative regulation of cyclin A degradation. The N-terminal ubiquitin-like domain is also shown to be essential for XDRP1 interaction with cyclin A. For example, Rad23, a protein containing the N-terminal ubiquitin-like domain, appears to interact with a subunit of XP-C to stabilize the repair complex XP-C (Reardon *et al.*, 1996; Schaubert *et al.*, 1998). So, as shown previously for XP-C, XDRP1 containing a ubiquitin-like sequence in its N-terminus might function as a molecular chaperone of the cyclin-CDK complex to protect the cyclin A substrate.

Several reports imply that mitotic cyclins must be protected from its degradation machinery under physiological conditions, even in a state of active proteasome in mitosis (Hunt *et al.*, 1992; Kobayashi *et al.*, 1994; Chen *et al.*, 1997). However, it is not yet clear whether the protection of cyclin A from degradation is of any biological significance. We are currently investigating this point, including other potential binding partners of XDRP1 in two-hybrid screens.

Materials and methods

Cloning of XDRP1

The N-terminal fragment of cyclin A1 (1–160) was obtained by PCR method using pGEM1-cyclin A1 (Minshull *et al.*, 1990) as a template and two oligonucleotides (5'-GATACATATGCGGCGCAGTATGGCTT-CCA-3' and 5'-TATGGATCCTTAAGCTACAGCATCAGGGTCT-3') as primers. The DNA fragment was digested with *Nde*I and *Bam*HI and subcloned into the multi-cloning site of PAS404 (a gift from E. Noguchi)

to obtain PAS404-A1 (1–160). *Saccharomyces cerevisiae* strain Y190 (a gift from N. Hayashi) was transformed with PAS404-A1 (1–160) linearized with *Xba*I. The expression of N-terminal cyclin A1 in the transformant was checked by immunoblotting. The transformant was then retransformed with *Xenopus* oocyte Matchmaker cDNA library (unfertilized oocytes from *Xenopus laevis*, ages 1–3 years; injected with a gonadotropin library, cloned into pGAD10 vector, Clontech). Fifty-three colonies that could grow on synthetic medium minus histidine plus 25 mM 3-aminotriazole were obtained from 1.8×10^8 transformants. Of those, 41 expressed β -galactosidase by filter assay as described in Clontech Matchmaker user manual. The partial sequence analysis of these candidates using a GAL4ADSP primer (5'-AATACCACTACA-ATGGATG-3') revealed 20 to be identical, and termed this clone XDRP1. A 2.3 kb stretch of full-length XDRP1 cDNA covers a 5' UTR, an ATG initiation codon, a complete single ORF (1755 nucleotides) encoding 585 amino acids, two stop codons, a 3' UTR and a polyadenylation signal at the 3' end. We used this clone for further analysis (DDBJ/EMBL/GenBank accession No. AB030502). An alternative spliced form of XDRP1 consisting of 600 amino acid residues was obtained by the screening of *Xenopus* cDNA library (5'-Stretch, oocyte from *X. laevis*, λ gt10; Clontech).

Purification of bacterially expressed GST-XDRP1 protein

A PCR fragment of XDRP1 was obtained using two oligonucleotides (5'-AAGAATTCTCATGGCGGAGAGCGGCGCTGAT-3' and 5'-TGA-GATGGTGCACGATGCAC-3') as primers and XDRP1 cDNA as a template. The DNA fragment was digested with *Eco*RI and subcloned into the multi-cloning site of pGEX-KG vector. The construct was used to transform the *Escherichia coli* strain BL21(DE3). The mid-log phase culture was induced with 0.5 mM IPTG and was grown at 26°C for 6 h. The cells were pelleted by centrifugation and frozen at -20°C. The pellet was thawed and suspended in lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 10% glycerol, 0.25% Tween 20, 1 mM DTT, 1 mM APMSF). The cells were broken by sonication at a medium intensity for 10 min. The lysate was centrifuged in a Beckman TLA-100 rotor at 45 000 r.p.m. for 45 min. The supernatant was incubated with glutathione-Sepharose 4B (Pharmacia Biotech) for 2 h at 4°C with rotating. The Sepharose beads were washed three times with lysis buffer and then resuspended and washed with ATP buffer [50 mM Tris-HCl pH 7.5, 10 mM Mg(OAc)₂, 100 mM KOAc, 10 mM ATP] and incubated at 30°C for 20 min. Then the beads were washed with beads-wash buffer (50 mM Tris pH 7.5, 150 mM NaCl, 50 mM DTT, 1 mM APMSF, 0.25% Tween 20) before eluting with the elute buffer (50 mM Tris pH 7.5, 150 mM NaCl, 50 mM DTT, 1 mM APMSF, 0.25% Tween 20, 50 mM reduced glutathione). The eluate containing GST-XDRP1 protein was concentrated and the buffer was replaced into XB (10 mM K-HEPES pH 7.7, 50 mM KCl, 2 mM MgCl₂) using an Ultrafree-4 centrifugal filter Biomax NMWL membrane (Millipore) before freezing in liquid nitrogen and storing at -80°C. For microinjection, GST-XDRP1 and GST were dissolved in 10 mM HEPES pH 7.7 and 88 mM NaCl. The GST-XDRP1 protein prepared by this method demonstrated a purity of ~90% or more.

Xenopus extracts

Xenopus oocytes and embryos, and the CSF-arrested extracts and interphase extracts were prepared by the standard methods of Murray's protocol (Murray, 1991). For activation of *Xenopus* eggs, squeezed eggs were treated by 2 μ g/ml of ionophore A23187 and the activated eggs were incubated at 20°C in 0.1 $\times$ MMR. At 10 min intervals, eggs were sampled (equivalent to one-tenth egg) and subjected to SDS-PAGE and immunoblotting with anti-XDRP1 antibody.

Antibodies, immunoblotting and immunoprecipitation

Bacterially expressed GST-XDRP1 was cleaved by thrombin and XDRP1 protein was recovered through elution from GST-Sepharose beads. This protein was used as an antigen for raising anti-XDRP1 antiserum in rabbits. To apply an affinity purification of anti-XDRP1 antibody, XDRP1 protein was adsorbed to a HiTrap affinity column (1 ml, Amersham Pharmacia Biotech). Anti-XDRP1 antibody bound to XDRP1 protein in this affinity column was eluted by 100 mM glycine, pH 2.8. The eluate was neutralized by 1 M Tris-HCl, pH 8.0. Anti-XDRP1 antiserum, diluted at 1:3000, was used for immunoblotting. *Xenopus* egg extracts labelled with [³⁵S]methionine (1 μ Ci/ μ l) were immunoprecipitated by affinity purified anti-XDRP1 antibody and the precipitates were analysed by SDS-PAGE, followed by autoradiography. The monoclonal *Xenopus* anti-cyclin B2 antibody and *Xenopus* anti-cyclin A1 were a gift from J. Gannon, and rabbit polyclonal *Xenopus* anti-cyclin A1 antibody was

obtained from M.Carrington, University of Cambridge, Cambridge, UK. *Xenopus* anti-cyclin A2 was the gift of M.Howell, ICRF, London, UK.

XDRP1 binding in vitro

mCAP RNA capping kit (Stratagene) was used for *in vitro* transcription. pGEM1-cyclin A1, pGEM1-cyclin B1, pGEM1-cyclin B2, myc-tagged cyclin A1 (ESP11), ESP11-cyclin A2 and human cyclin A were used as templates. mRNAs were added at 0.1 µg/µl and 0.5 µCi of [³⁵S]methionine in 20 µl of the reaction mixed extracts [80% of reticulocyte lysate (Amersham) and 20% of *Xenopus* CSF-arrested extracts]. After incubation at 23°C for 40 min, the GST or GST-XDRP1 protein was added to the extracts and incubated at 23°C for 80 min. The reaction was diluted with 400 µl of RIPA buffer (50 mM Tris-HCl pH 7.5, 0.5% sodium deoxycholate, 0.1% SDS, 1% NP-40, 150 mM NaCl, 25 mM APMSF, 1 mM DTT) and 10 µl of glutathione-Sepharose beads was added and incubated for 1 h at 4°C with rotation. The beads were washed with RIPA buffer three times. Proteins bound to GSH beads were detected by SDS-PAGE and autoradiography.

Transfection and binding assay

Full-length XDRP1 and *Xenopus* cyclin A2 were subcloned into the multi-cloning site of vector pCDNA with N-terminal T7-His₆ tags and with N-terminal HA tag, respectively. To obtain pCDNA T7-His-XDRP1, the PCR product of pGAD10-XDRP1 as a template using oligonucleotides ATGGATCCATGGCGGAGAGCGGCGCTGAT and TGAGATGGTGCACGATGCAC was digested by *Bam*HI. The digested PCR fragment was subcloned into the *Bam*HI site of the plasmid pCDNA with T7-His tags. Similarly, to obtain pCDNA HA-cyclin A2, the PCR product of *Xenopus* pGEM1-cyclin A2 as a template using oligonucleotides GGTGGTATGCGGCCGATGTCTGACCATCTGCT-GCGG and TAAGATATCTTATAAAAATGTAGACATCGA was digested with *Nor*I and *Eco*RV. The PCR fragment was subcloned into *Nor*I and *Eco*RV sites of HA-tagged pCDNA (both HA-tagged pCDNA and T7-His₆ tagged pCDNA, were given by T.Sekiguchi).

Xenopus fibroblast WAK cells were cultured in a medium for *Xenopus* cell line (65% modified Eagle's medium, 10% fetal calf serum, 20 mM HEPES pH 7.2, 100 µg/ml penicillin and 100 µg/ml streptomycin) at 26°C (Kobayashi *et al.*, 1992). WAK cells were transfected with HA-tagged cyclin A2 and T7-His-tagged XDRP1 in pCDNA at ~80% confluence on a 10 cm dish using 60 µl of Superfect Transfection Reagent (Qiagen). These cells were incubated for 24 h at 26°C. The transfected cells (1 × 10⁷ cells) were lysed on ice in 1 ml of modified lysis buffer A (10 mM HEPES pH 7.5, 150 mM NaCl, 1 mM EGTA, 0.5% Triton X-100, 1 mM APMSF, 1 mg/ml leupeptin and 1 mg/ml aprotinin; Qian *et al.*, 1998) for 50 min on ice with occasional rocking. The lysates were clarified by centrifugation at 10 000 g for 15 min. The 900 µl of supernatants were incubated with 10 µl of TALON metal affinity resin (Clontech) for 2 h at 4°C and washed with modified lysis buffer A three times. 3× SDS-sample buffer was added to the beads and boiled for 3 min.

Immunofluorescence

Xenopus WAK cells were cultured asynchronously in the medium, as described, on coverslips overnight. The coverslips were rinsed in CMFM (88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO₃, 7.5 mM Tris-HCl, pH 7.6) three times at room temperature and fixed for 10 min in CMFM containing 4% paraformaldehyde for 10 min. Coverslips were washed with CMFM, then incubated with CMFM containing 1% Triton X-100 for 10 min. The coverslips were incubated with blocking solution [CMFM containing 5% gelatin (from cold water fish skin, Sigma), 1% Tween 20, 3% ovalbumin, 10% normal goat serum] for 1 h and incubated with affinity purified anti-XDRP1 antibody (1:100) for 1 h. After washing three times with CMFM containing 1% Tween 20, coverslips were incubated with secondary antibody (fluorescein conjugated-goat anti-rabbit antibody, Biosource International, 1:500). The secondary antibody was incubated on the fixed WAK cells to remove any background before use. After 45 min of incubation, the coverslips were washed with CMFM containing 1% Tween 20. Vectashield with DAPI (Vector Laboratories) was used for mounting on glass slides. Photographs were taken using a Neofluar 40× lens (Zeiss) on Kodak Ektachrome DNYA 400.

Subcellular fractionation

Xenopus WAK cells were removed by scraping and washing three times with CMFM buffer. Cell pellets were suspended in pre-chilled hypotonic buffer (10 mM Tris-HCl pH 7.4, 10 mM NaCl, 1.5 mM MgCl₂, 1 mM APMSF) and incubated on ice for 15 min. The cell suspension was homogenized with 10–20 strokes in a Dounce homogenizer and centri-

fuged at 1000 g for 5 min. The supernatant contained cytoplasm was centrifuged at 1000 g for 5 min to remove cell debris. The pellet contained nuclei was then washed with hypotonic buffer. Three micrograms of the fractionated proteins were analyzed by SDS-PAGE and immunoblotting. The proteins of nuclear fraction were prepared from 4-fold equivalent cells, compared with cytoplasmic ones.

Treatment by phosphatase and CDK inhibitor

The CSF-arrested extracts (3 µl) were added to 17 µl of the reaction mixture of λ-phosphatase (10 U/µl of λ-phosphatase, 50 mM Tris-HCl pH 7.5, 0.1 mM EDTA, 5 mM DTT, 0.01% Brij 35, 2 mM MnCl₂) and incubated at 30°C for 1 h in the presence or absence of phosphatase inhibitor cocktail (5 mM sodium fluoride, 5 mM sodium phosphate pH 8.0, 10 mM sodium pyrophosphate, 5 mM EGTA, 5 mM EDTA, 0.1 mM sodium orthovanadate; Randez-Gil *et al.*, 1998). To analyse the phosphorylation of purified GST-XDRP1 protein, cyclin A1 mRNA was translated in CSF/Retic-mixed extracts (1:1) at 23°C for 40 min, and then 1 µg of GST-XDRP1 protein was added. After incubation for 80 min, GST-XDRP1 protein was retained with glutathione-Sepharose beads, and treated in 20 µl of the reaction containing 10 U/µl of λ-phosphatase as described above. The bead-bound GST-XDRP1 was detected by immunoblotting with anti-XDRP1 antibody. For CDK inhibitory assay, cyclin A1 mRNA was translated in CSF/Retic-mixed extracts (1:1) at 23°C for 40 min, and then 1 µg of GST-XDRP1 protein and the increasing amounts of butyrolactone I (provided by M.Kitagawa) were added to the extracts. After incubation for 60 min, GST-XDRP1 was detected by immunoblotting with anti-XDRP1 antibody.

Histone H1 kinase assay

Xenopus cyclin A1 mRNA was translated in CSF/Retic-mixed extracts (1:1) with [³⁵S]methionine at 23°C for 40 min, and GST-XDRP1 was added to the extracts and incubated for 80 min. GST-XDRP1 was recovered by the GSH beads as described above. The beads were washed with the washing buffer (20 mM Tris-HCl, 7.5 mM MgCl₂) and then incubated with reaction mixture (20 mM Tris-HCl pH 7.5, 7.5 mM MgCl₂, 0.2 mM ATP, 10 µCi [γ-³²P]ATP, 100 µg/ml histone H1) for 30 min at 23°C. The samples were analyzed by SDS-PAGE and autoradiography (overnight exposure).

Degradation assay and immunodepletion

³⁵S-labelled cyclin A1 and cyclin B2 as the substrate were prepared by *in vitro* translation in the mixed extracts (50% reticulocyte lysate and 50% CSF-arrested extracts) and added to the pure CSF-arrested extracts. Normally, 1–2 µl of the extracts containing labelled cyclin A1 and cyclin B2 was added to 15 µl of pure CSF-arrested extracts. GST or GST-XDRP1 protein was added. CaCl₂ was then added to 0.4 mM final concentration and the samples was taken at the indicated times and were analysed by SDS-PAGE and autoradiography. The addition of at least 50 ng/µl of GST-XDRP1 to the extracts exhibited an inhibitory effect on the degradation of cyclin A.

For immunodepletion, protein A beads (Affiprep, Bio-Rad) were pre-coated with affinity purified anti-XDRP1 antibody (2 mg/ml) and washed in XB buffer. The beads were added to CSF-arrested extracts (20% v/v) containing 250 µg/ml cycloheximide, and incubated for 1 h at 4°C with rotation. The beads were removed by centrifugation and the supernatants were used as depleted extracts.

Microinjection of Xenopus embryo

Unfertilized eggs were inseminated with sperm, and then dejellied in 0.3× MBS (Gurdon, 1991) containing 2% cysteine for 5 min. Dejellied embryos were transferred to 0.3× MBS containing 3% Ficoll (Type 400-DL) for microinjection. GST or GST-XDRP1 protein (60 ng each) was injected into embryos at one-cell stage (50–60 min after fertilization). The injected embryos were allowed to develop in 0.3× MBS medium. These embryos were fixed with 5% trichloroacetic acid for 30 min and photographed at 3 h after injection.

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