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A member of the Ste20/PAK family of protein kinases is involved in both arrest of *Xenopus* oocytes at G_2/prophase of the first meiotic cell cycle and in prevention of apoptosis

Sandrine Faure, Suzanne Vigneron, Marcel Dorée and Nathalie Morin[1]

Centre de Recherches de Biochimie Macromoléculaire, CNRS ERS 155, 1919 route de Mende, 34293 Montpellier Cedex 5, France

[1]Corresponding author
e-mail: doree@crbm.cnrs-mop.fr

This paper is dedicated to Jean-Claude Cavadore, who passed away on March 19, 1997

We have identified new members (X-PAKs) of the Ste20/PAK family of protein kinases in *Xenopus*, and investigated their role in the process that maintains oocytes arrested in the cell cycle. Microinjection of a catalytically inactive mutant of X-PAK1 with a K/R substitution in the ATP binding site, also deleted of its Nter-half that contains the conserved domains responsible for binding of both Cdc42/Rac GTPases and SH3-containing proteins, greatly facilitates oocyte release from G_2/prophase arrest by progesterone and insulin. Addition of the same X-PAK1 mutant to cell cycle extracts from unfertilized eggs induced apoptosis, as shown by activation of caspases and cytological changes in *in vitro*-assembled nuclei. This was suppressed by adding Bcl-2 or the DEVD peptide inhibitor of caspases, and rescued by competing the dominant-negative mutant with its constitutively active X-PAK1 counterpart. Such results indicate that X-PAK1 (or another member of the *Xenopus* Ste20/PAK family of protein kinases) is involved in arrest of oocytes at G_2/prophase and prevention of apoptosis; thus death by apoptosis and release of healthy oocytes from cell cycle arrest may be linked. That cell cycle arrest protects oocytes from apoptosis is consistent with the finding that extracts from metaphase II-arrested oocytes are less sensitive to apoptotic signals than those from activated eggs.

Keywords: apoptosis/cell cycle arrest/meiotic maturation/oocyte release/X-PAK1

Introduction

Quiescent cells have been reported to be poorly sensitive to apoptosis, while many signals that stimulate proliferation may also induce a cell to enter apoptosis. None the less, relationships between cell cycle and apoptosis remain poorly understood (for review, see Evan *et al*., 1995) in spite of potential implications for the chemotherapeutic treatment of cancers.

Oocytes are cells that naturally arrest for long periods of time at G_2 in the ovary, and release of G_2 arrest has been shown to be associated with apoptosis in some somatic cells (Abend *et al*., 1996; Sit and Chen, 1997). A small number of oocytes in the female germline will ultimately undergo fertilization and successful development, but the vast majority will die. The fate of oocytes in the ovary depends on follicle cells that enclose and nourish them until their release at ovulation. Follicle cells provide cytokines, such as IGF-1, that are essential to the survival of oocytes in culture. They also provide inhibitory signals that keep oocytes arrested in the cell cycle, for several years in many species. Conversely, disrupting the follicle is sufficient to induce the previously enclosed mammalian oocyte to escape arrest at first meiotic prophase and to undergo meiotic maturation *in vitro* (Edwards, 1965). However, oocytes frequently exhibit germinal vesicle breakdown *in vivo* in atretic follicles (Gougeon and Testart, 1986). When oocytes initiate meiotic maturation under physiological conditions, due to some change in the balance of systemic regulatory mechanisms, contacts between follicle cells and the oocyte are disrupted, suppressing the conveyance of both survival and antiproliferative factors. Besides the well-known relationships between meiotic maturation and successful fertilization (reviewed in Masui and Clarke, 1979), there should thus exist some link between the release from cell cycle arrest and the death of a majority of oocytes.

How contacts with follicle cells can contribute to the arrest of oocytes at the G_2/prophase boundary of the meiotic cell cycle has not been thoroughly investigated. A likely possibility is that these inhibitory contacts may involve in the oocyte plasma membrane G-protein-coupled components of the serpentine family of receptor proteins. Indeed, there is some evidence that heterotrimeric G-proteins may mediate the effect of hormones, progesterone and 1-methyladenine, both synthesized within follicle cells, and responsible for meiosis reinitiation in amphibian and starfish oocytes respectively, through interaction with plasma membrane receptors (Chiba *et al*., 1993; Gallo *et al*., 1995). On the other hand, hormonal signals that induce oocyte maturation have been proposed to prime follicle-cell-enclosed oocytes for apoptosis, and this provided the rationale for the development of a cell-free assay to investigate apoptotic mechanisms in *Xenopus* oocytes (Newmeyer *et al*., 1994).

Another example of G-protein-associated serpentine receptor responsible for cell cycle arrest is the pheromone receptor of budding yeast. In this case, binding of pheromones to receptors releases $G\beta\gamma$ from the heterotrimeric complex, and the signalling subunit activates a MAP kinase cascade through the action of Ste20 protein kinase (Leberer *et al*., 1992). Ste20-like kinases are not unique to yeast: homologues have been discovered in a wide variety of mammalian systems. For example, human PAK1 is able to functionally complement Ste20 in yeast (reviewed in Brown *et al*., 1996).

In the present work, we have identified new members

of the Ste20/PAK family of protein kinases in *Xenopus* oocytes, and investigated their possible role in the process that maintains oocytes arrested in the cell cycle. Specifically, we found that microinjection of a catalytically inactive mutant of X-PAK1, a *Xenopus* Ste20/PAK homologue, facilitates escape of progesterone-treated oocytes from cell cycle arrest. As release of oocytes from G_2 arrest has been proposed to prime them for apoptosis, and MAP kinase families downstream of human PAKs are suggested to regulate apoptosis (Xia *et al*., 1995; Zhang *et al*., 1995; Teramoto *et al*., 1996; Tibbles *et al*., 1996). We also investigated the possible involvement of *Xenopus* homologues in this process and found that catalytically inactive X-PAK1, acting as a dominant-negative mutant, readily induces apoptosis in *Xenopus* egg extracts.

Results

Identification of Ste20/PAK homologues in Xenopus oocytes

Recently, a number of groups have pointed out the growing importance of MAP kinase cascade regulation by Ste20/PAK kinase family members (reviewed in Kyriakis and Avruch, 1996). Because of our interest in the regulation of oocyte maturation in *Xenopus*, we decided to search for *Xenopus* homologues of p21-activated kinase. PCRs were performed with degenerate oligonucleotides designed in the conserved kinase domain. Single-strand cDNAs prepared from mRNAs from metaphase II-arrested eggs were used as a template. Several different clones sharing a strong homology to known PAK proteins were isolated, indicating that *Xenopus* PAKs (X-PAK) are likely to belong to a kinase family. Of these clones, three were chosen to screen a λgt10 library from stage 6 oocytes. A full-length clone was isolated using the first probe, and its deduced amino acid sequence encodes for a 524-amino acid protein, which we have named X-PAK1. Two other full-length sequences encoding for X-PAK2 and X-PAK3 were reconstructed by a combination of screening and 5′ RACE PCR techniques. X-PAK1, 2 and 3 share overall identities to human PAK1 (Manser *et al*., 1994) of 71%, 88% and 41% respectively (data not shown). In this report, we wish to focus on X-PAK1 characterization.

The N-terminal region of X-PAK1 (Figure 1) is likely to be a regulatory domain as it bears both a putative Cdc42/Rac1 binding site (starting at position 64) and three potential SH3 binding sites (starting at positions 162/173/194). The C-terminal region contains the XI kinase subdomains identified by Hanks and Hunter (1995), as well as—C-terminal to the catalytic domain—a small peptide SLTPYI which is conserved in most known PAK sequences. In this regard, it is interesting to note recent results on the role played by the kinase domain of Ste20p and its C-terminal tail in pheromone signalling, independently of the Cdc42p binding site (Peter *et al*., 1996; Leberer *et al*., 1997).

Antibodies developed against the specific C-terminal peptide GKQIAKGGH (not conserved in other members

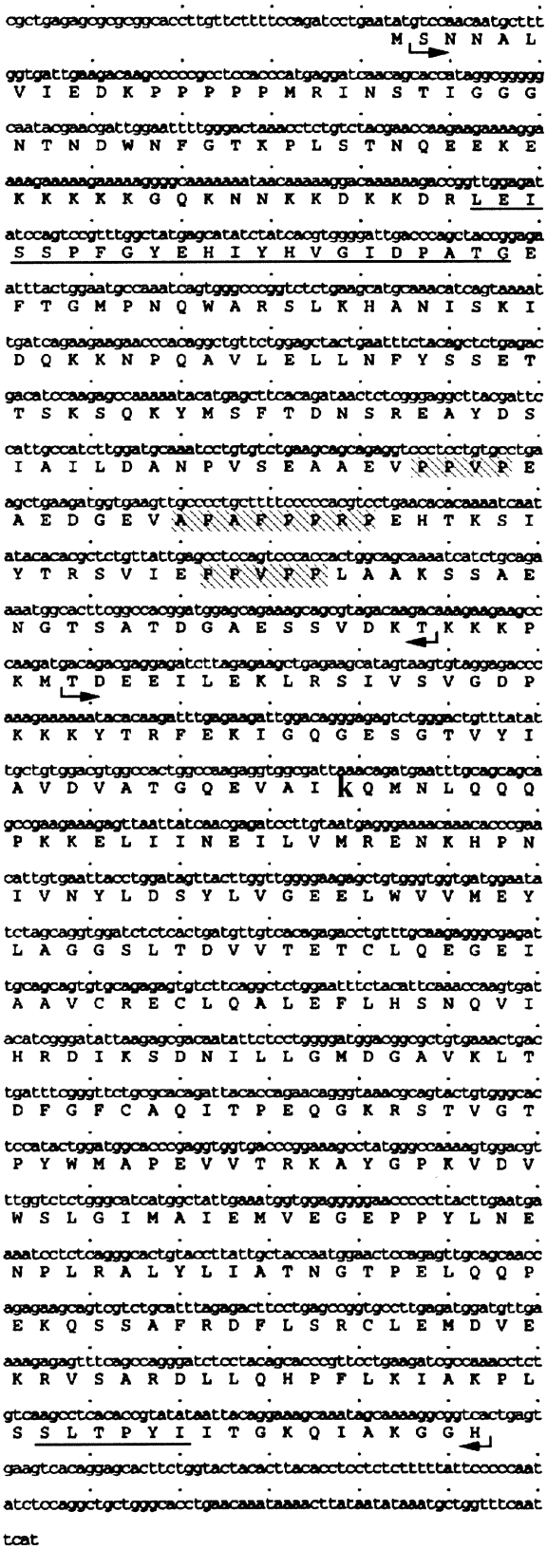

Fig. 1. Nucleotide and predicted amino acid sequence of *Xenopus* X-PAK1. The underlined N-terminal 22 amino acid sequence corresponds to the putative Cdc42/Rac1 binding site, the three dashed amino acid sequences to potential SH3 binding sites, and the underlined C-terminal six amino acid sequence to a motif conserved in most known PAK sequences, the function of which is as yet unknown. Lys279 (in bold) is located in the putative ATP binding site. The substitution K/R abolished the kinase activity of X-PAK1. The two domains enclosed by arrows show the X-PAK1 N-terminal and C-terminal domains which are expressed in pMal-p2 vectors. The sequence of X-PAK1 has been submitted to DDBJ/EMBL/Genbank under the accession number AF 000 239.

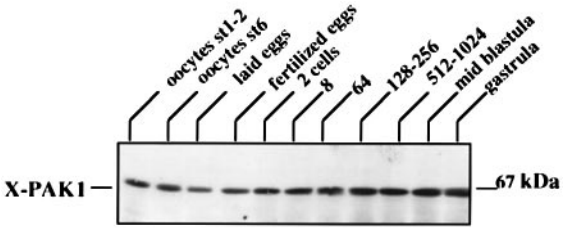

Fig. 2. Western blot analysis of X-PAK1 in protein extracts prepared from *Xenopus* oocytes through oogenesis (stages 1–2; stage 6; unfertilized laid eggs) and from *Xenopus* embryos through early development (from fertilization to gastrulation). Identical amounts of proteins (150 μg) were separated by SDS–PAGE before Western blot analysis.

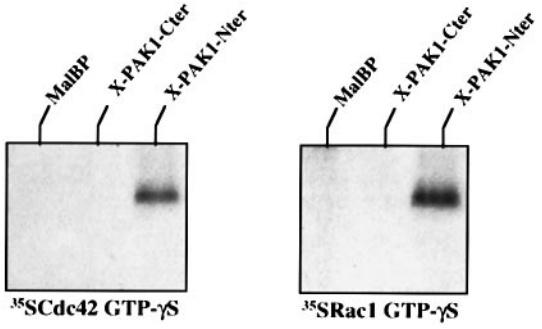

Fig. 3. Cdc42 and Rac bind to the N-terminal region of X-PAK1. The N-terminal (residues 2–222) and the C-terminal (residues 229–524) domains of X-PAK1 were expressed as fusion proteins with Mal-BP. Samples (10 μg) of Mal-BP, X-PAK1-Cter and X-PAK1-Nter were separated by SDS–PAGE, Western-blotted on PVDF membranes and overlaid with [γ-^{35}S]GTP-labelled Cdc42 (left panel) or Rac1 (right panel): both GTPases bind only to X-PAK-1-Nter.

of the *Xenopus* X-PAK family) allowed us to study the expression of X-PAK1 during early embryogenesis. Western blot analysis, using purified antibodies, of protein extracts made from small oocytes (stage I and II), stage VI oocytes, eggs and embryos up to the gastrula stage (Gerhart, 1980), show that X-PAK1 is present at a comparable level throughout oogenesis and early embryogenesis (Figure 2). X-PAK1 migrates as a single band of apparent mol wt 65 kDa, which co-migrates with the *in vitro*-translated [^{35}S]methionine-labelled X-PAK1 full-length clone (data not shown). No shift of the electrophoretic mobility of X-PAK1 could be detected during early development.

In order to characterize X-PAK1 domains further, we expressed the N-terminal regulatory domain (X-PAK1-Nter, residues 1–222), the C-terminal half comprising the kinase domain (X-PAK1-Cter, residues 229–524) and a mutant of the latter with a substitution K/R at position 279 in the putative ATP binding site (X-PAK1-Cter K/R) as fusion proteins with maltose-binding protein (Mal-BP). Purified X-PAK1-Nter, X-PAK1-Cter, and Mal-BP alone were tested for their ability to bind in overlay assays the small GTPases Cdc42 and Rac1 loaded with [γ-^{35}S]GTP. As expected, Figure 3 shows that X-PAK1-Nter can bind both Rac1 and Cdc42, while neither X-PAK1-Cter nor Mal-BP control are capable of any detectable interaction with the small GTP-binding proteins.

The constitutive catalytic activity of the C-terminal half of X-PAK1, deleted of its N-terminal regulatory region, was tested by in-gel kinase assay. The mutant with a K/R

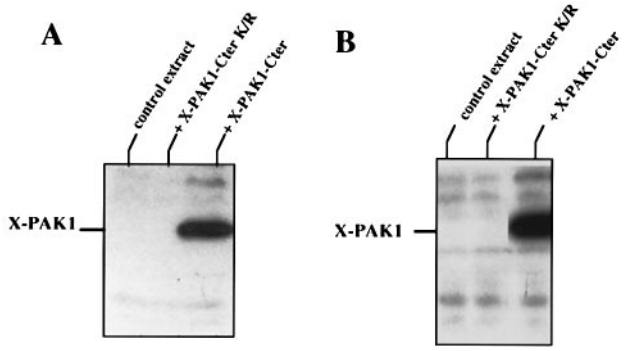

Fig. 4. Constitutive kinase activity of X-PAK1-Cter. Recombinant X-PAK1-Cter and X-PAK1-Cter K/R Mal-BP fusion proteins were tested for their ability to autophosphorylate (**A**) or to phosphorylate exogenous substrate MBP (**B**) by in-gel kinase assay. A *Xenopus* extract was incubated at room temperature for 30 min without any addition (control), with X-PAK1-Cter, or with X-PAK1-Cter K/R. 2 μl of extract were loaded on SDS–PAGE containing no substrate (A) or MBP at 0.5 mg/ml (B). After the run, gels were subjected to a cycle of denaturing and renaturing conditions, then incubated with [γ-^{32}P]ATP, washed, dried and processed for autoradiography.

substitution in the putative ATP binding site was tested in parallel as a further control. Both autophosphorylation (Figure 4A) and phosphorylation of the exogenous substrate Myelin Basic Protein (MBP) (Figure 4B) could be observed after incubation, for 30 min, of X-PAK1-Cter in a *Xenopus* extract (Figure 4, right-hand lanes). However, no signal could be detected when the recombinant constitutive kinase was omitted (left-hand lanes) or replaced by its K/R mutant (centre lanes), thus confirming that the K/R mutation abolished the kinase activity, resulting in a kinase-dead mutant.

A catalytically inactive mutant of X-PAK1 facilitates progesterone-induced meiotic maturation independently of Cdc42/Rac

The small Rho family GTPase Cdc42 has been proposed to be an essential signalling element responsible for activation of Ste20 in response to stimulation of the pheromone seven transmembrane receptor in budding yeast (Simon *et al*., 1995; Zhao *et al*., 1995). We showed, in the preceding section, that a bacterially-expressed polypeptide encoding the N-terminal segment of X-PAK1 in fusion with Mal-BP binds human Cdc42 and Rac1 GTPases efficiently. As Cdc42 and Rac proteins are highly conserved in vertebrates, this polypeptide could be expected to bind *Xenopus* Cdc42 and Rac proteins at least as efficiently.

To investigate the possible involvement of these G-proteins in the release of oocytes from G_2 arrest, X-PAK1-Nter was microinjected to the final concentration of 20 μg/ml, and microinjected oocytes were scored for germinal vesicle breakdown (GVBD) as a function of time in the absence or presence of progesterone. As shown in Figure 5, the recombinant protein neither induced meiotic maturation by itself, nor modified the kinetics of GVBD due to progesterone.

As the amount of microinjected protein was in excess of endogenous X-PAK 1, and sufficient to titrate as much as 5 pmol of Cdc42/Rac proteins per microinjected oocyte (not shown), the above results make it very unlikely that small GTPases of the Cdc42/Rac subfamily are required

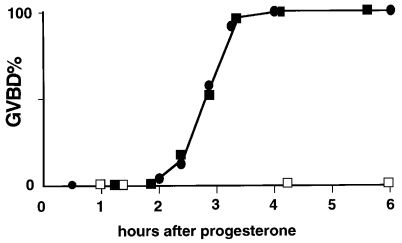

Fig. 5. Time course of germinal vesicle breakdown (GVBD) following progesterone stimulation (10 μg/ml) of control non-injected oocytes (●) or of oocytes injected with the recombinant X-PAK1-Nter protein at the final intracellular concentration of 20 μg/ml (■). Microinjected oocytes non-treated with progesterone failed to undergo GVBD at any time (□). A total of 84 microinjected oocytes were scored for this experiment.

for release of *Xenopus* oocytes from G_2 arrest in response to progesterone. The recombinant X-PAK1-Nter polypeptide contains specific proline-rich sequences that are potential SH3-binding sites (Figure 1). Although we did not directly check the ability of X-PAK1-Nter to bind proteins containing SH3 domains, our experiments suggest that SH3 domains may not be essential signalling elements of *Xenopus* oocytes in response to progesterone, confirming previous results (Pomerance *et al*., 1996).

Recently, it was shown that the conserved Cdc42 binding domain of Ste20 is dispensable for heterotrimeric G-protein-mediated pheromone signalling in budding yeast (Peter *et al*., 1996; Leberer *et al*., 1997). In fact a N-terminally deleted Ste20/PAK mutant was shown still to be regulated by Gβγ in yeast, demonstrating that molecular determinants which specify regulation of Ste20/PAK by Gβγ lie within either the kinase domain or, more likely, the non-catalytic sequence C-terminal to the kinase domain (Peter *et al*., 1996; Leberer *et al*., 1997). This led us to examine, in spite of the above negative results, the possible effect of microinjecting the N-terminally deleted, constitutively active X-PAK1-Cter recombinant protein kinase (in fusion with Mal-BP) on meiotic maturation of *Xenopus* oocytes. When microinjected at a final intracellular concentration of <5 μg/ml, the recombinant protein had no detectable effect, either in the absence or in the presence of progesterone (not shown). In contrast, oocytes microinjected with the X-PAK1-Cter recombinant protein at a final intracellular concentration of 20 μg/ml were found to be completely refractory to stimulation by progesterone, as they failed to activate Maturation Promoting Factor (MPF) and remained arrested at G_2, even when the hormone was used at 10 μg/ml (Figure 6A). Oocytes microinjected with 20 μg/ml of the X-PAK1-Cter recombinant protein were also found to suppress meiotic maturation by insulin (Figure 7A). Suppression of meiotic maturation was not due to irreversible damage of oocytes following microinjection of a relatively large amount of protein of bacterial origin (~0.3 μM, final intracellular concentration) for two reasons. First, microinjection of an even larger amount (50 μg/ml) of recombinant Mal-BP had no effect on hormone-induced meiotic maturation. Second, part of the oocytes microinjected with X-PAK-Cter were observed in some experiments (not shown) to escape the block after overnight incubation in the presence of progesterone.

The above results suggested that an endogenous X-PAK

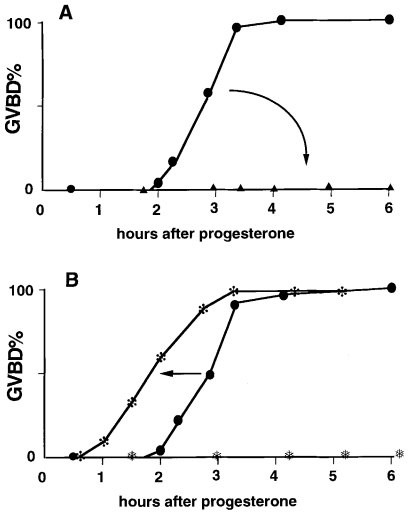

Fig. 6. Time course of GVBD following progesterone stimulation (10 μg/ml) of control non-injected oocytes (●) or oocytes injected with (**A**) the recombinant X-PAK1-Cter protein (▲) or (**B**) the recombinant X-PAK1-Cter K/R mutant protein (❊), both at the final intracellular concentration of 20 μg/ml. In this experiment, oocytes microinjected with X-PAK1-Cter K/R and not treated with progesterone (❊) (B) failed to undergo GVBD. A total of 108 microinjected oocytes were scored for these experiments.

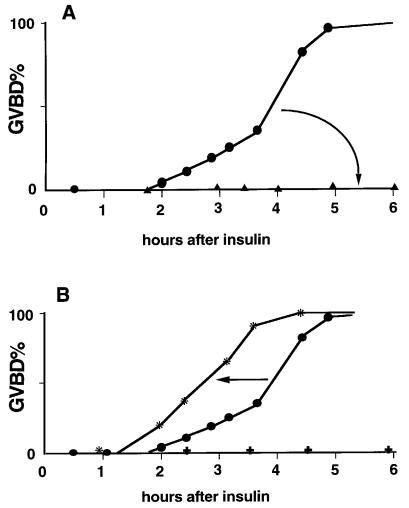

Fig. 7. Time course of GVBD following insulin stimulation (1 μM) of control non-injected oocytes (●) or oocytes injected with (**A**) the recombinant X-PAK1-Cter protein (▲) or (**B**) the recombinant X-PAK1-Cter K/R mutant protein (❊), both at the final intracellular concentration of 20 μg/ml. In this experiment, oocytes microinjected with X-PAK1-Cter K/R and not treated with insulin (+) (B) failed to undergo GVBD. A total of 95 microinjected oocytes were scored for these experiments.

homologue may contribute to cell cycle arrest at the first meiotic G_2/prophase boundary by inhibiting some step essential for meiotic maturation in the cascade of common events triggered by both insulin and progesterone.

If X-PAK1, or possibly another member of the same family, is indeed involved in arrest of oocytes at G_2, the microinjection of an inactive form of the kinase as its X-PAK1-Cter K/R mutant (see Figure 4) should either induce GVBD by itself, or facilitate exit from G_2 arrest due to either progesterone or insulin action. X-PAK1-Cter K/R was indeed found to induce maturation by itself in a few batches of oocytes. In most cases, however, it

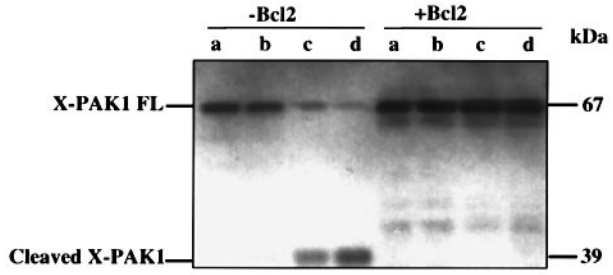

Fig. 8. Time course of full-length X-PAK1 cleavage in *Xenopus* eggs extract undergoing apoptosis. An apoptotic egg extract was incubated in the presence of Mock- or Bcl2-transfected Cos cells lysate for 15 min before addition of [^{35}S]methionine-labelled full-length (FL) X-PAK1 generated by *in vitro* transcription/translation. 3 µl samples were removed at 60 (a), 90 (b), 150 (c), 210 (d) min and analysed by SDS–PAGE and autoradiography. A 39 kDa X-PAK1 proteolytic fragment is generated during the course of apoptosis.

was found reproducibly to speed up GVBD following either progesterone (Figure 6B) or insulin addition (Figure 7B). The time for $GVBD_{50}$ was reduced by between 35–65% and this was consistently associated with earlier activation of MPF as compared with controls (not shown). Thus, the addition of the mutated kinase led to a dominant-negative effect, presumably because the protein titrates the partners and/or the substrates of X-PAK1, or a related kinase. The titrated component is certainly not a member of the Cdc42/Rac subfamily of GTPases or a protein with SH3 domains, as microinjection of the N-terminal fragment of X-PAK1, that binds such components, had no effect on meiotic maturation. Possible candidates may include heterotrimeric G-proteins, as Gβ was claimed to bind directly Ste20/PAK in budding yeast (Leberer *et al*., 1997). However, it remains unclear whether Gβ interacts directly with members of the Ste20/PAK family in vertebrates, even though several isoforms have been shown to be regulated downstream of G-protein-coupled receptors (Knaus *et al*., 1995; Teo *et al*., 1995).

As the K/R mutant of X-PAK1-Cter has no kinase activity (Figure 4), the above results strongly suggest that, even deleted from its well-documented N-terminal regulatory domain, it binds both GTPases of the Cdc42/Rac subfamily and, probably, proteins with SH3-domains; and that the X-PAK1-Cter K/R mutant could titrate some component of the oocyte machinery that contributes to cell cycle arrest at the first meiotic G_2/prophase boundary.

Recently, Newmeyer *et al*. (1994) reported the development of an *in vitro* assay derived from *Xenopus* eggs that support activation of caspases and reproduce apoptotic events *in vitro*. As shown in Figure 8, in experiments using ^{35}S-labelled *in vitro* translated full-length X-PAK1, we showed that full-length X-PAK1 is cleaved in *Xenopus* egg extracts undergoing the apoptotic process, and that this cleavage was suppressed if the extract was pre-incubated with a Bcl-2-transfected Cos cell lysate. Cleavage of endogenous X-PAK1 during apoptosis was also detected by Western immunoblotting (data not shown). As we were interested in mechanisms that may possibly link death by apoptosis of most ovarian oocytes with their release after months or years from cell cycle arrest (see Introduction), we investigated—as reported later—the possibility that this link may involve a member of the X-PAK family.

The catalytically inactive X-PAK1 mutant triggers apoptosis in Xenopus egg extracts, independently of its N-terminal regulatory domain

The localization of Bcl-2 in the outer mitochondrial membrane (Monaghan *et al*., 1992; Krajewski *et al*., 1993; de Jong *et al*., 1994) suggested a role for mitochondria in apoptosis. Newmeyer *et al*. (1994) reported in *Xenopus* egg extracts that efficient induction of apoptosis *in vitro* depends on a heavy membrane-containing fraction, mainly mitochondria. According to the authors, to lay eggs suitable for apoptotic extracts, the toads should be primed *in vivo* to start the atretic programme by allowing a long interval between the hormonal stimulus for oocyte maturation and induction of ovulation.

As emphasized by Liu *et al*. (1996), cell-free systems derived from cells that were already engaged in the apoptotic programme suffer the disadvantage that they cannot be used to detect triggering factors. In contrast to Newmeyer *et al*., however, we found that readily fertilizable eggs, not primed to undergo apoptosis, can be used to prepare a cell-free system that induces apoptotic changes in the added nuclei. In fact, even the widely used egg extracts developed by Murray (1991) to reconstitute cell cycle processes *in vitro* can be induced to undergo apoptosis reproducibly simply by increasing the ratio of the heavy membranes to the cytosol fraction. Cell cycle extracts, prepared by recovering, after centrifugation at 15 000 *g*, both the supernatant and the full membrane fraction (that contain both mitochondria and other membrane vesicles), do not normally enter apoptosis but rather assemble from permeabilized sperm heads, healthy nuclei that can be induced to assemble functional spindles (Figure 9B). If the amount of mitochondria is increased 2-fold, however, by adding a concentrated suspension of purified mitochondria prepared from the same eggs (see Materials and methods), the *in vitro*-assembled nuclei undergo fragmentation and form characteristic apoptotic bodies (Figure 9A).

Even though they are useful to visualize apoptotic events in cell cycle extracts receiving excess mitochondria, added nuclei are unnecessary for apoptosis to occur. Indeed, the *in vitro*-translated and [^{35}S]methionine-labelled *Xenopus* poly-ADPR polymerase (PARP, 113 kDa) readily generated a conserved proteolytic fragment of 89 kDa (de Murcia *et al*., 1994), after adding the mitochondrial fraction, even in the absence of added nuclei (Figure 10). In the same way, degradation of [^{35}S]methionine-labelled mammalian procaspase YAMA (CPP32) was found to yield proteolytic fragments of 20, 16 and 12 kDa (Fernandes-Alnemri *et al*., 1994; Tewari *et al*., 1995), respectively (Figure 10). As noted previously by Cosulich *et al*. (1996), proteolysis of PARP occurred a few minutes before that of CPP32, suggesting that a *Xenopus* caspase which degrades PARP is activated before that responsible for CPP32 activation in the cascade of apoptotic events. Proteolysis of both PARP and CPP32 (Figure 10) could be prevented by adding a COS cell lysate containing Bcl-2 or the DEVD peptide inhibitor of caspases (Nicholson *et al*., 1995) before the mitochondrial fraction.

Our data show that specific priming of the toads, in order to induce atresia, is not required to prepare apoptotic extracts. The latter can readily be made from unprimed laid eggs and thus are suitable to screen triggering factors.

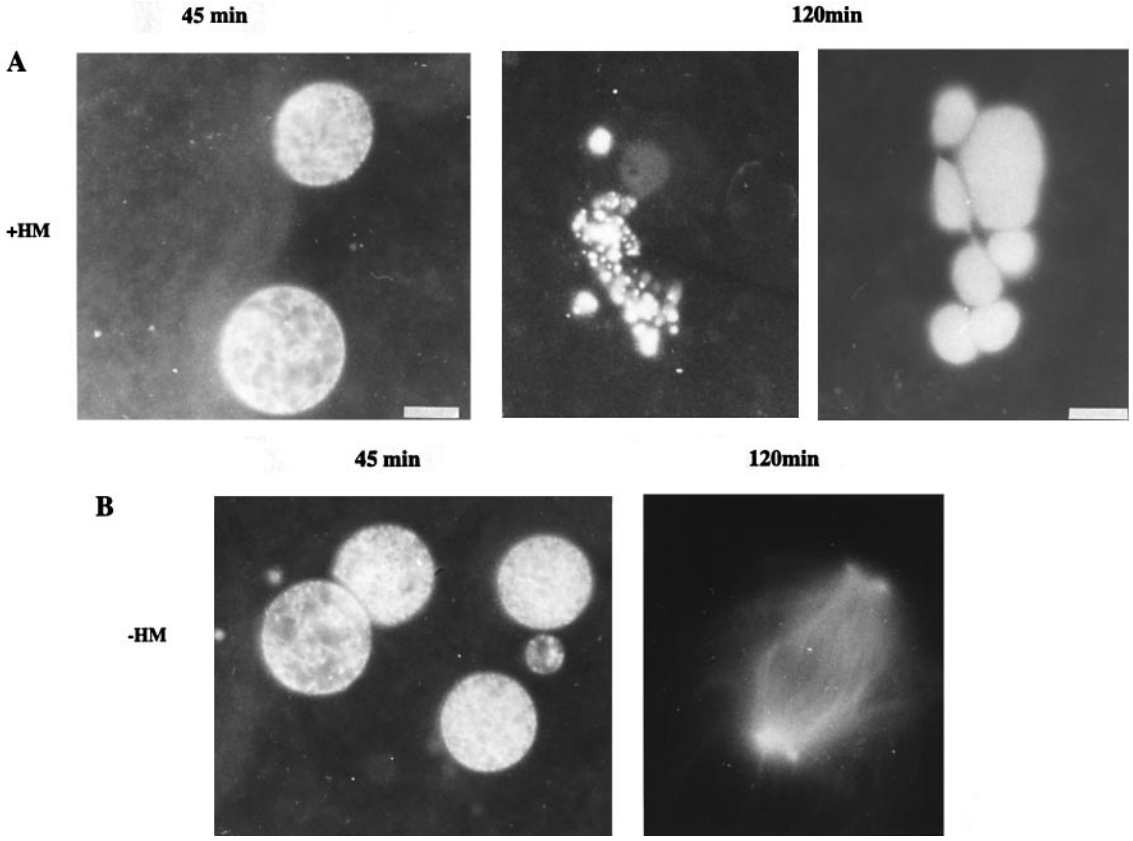

Fig. 9. *In vitro*-assembled nuclei form apoptotic bodies in *Xenopus* egg extracts to which a heavy membrane fraction (HM) has been added. (**A**) Sperm heads were added at zero time to an extract to which a HM fraction was added. Samples were collected after 45 min (left micrograph) and 120 min (middle and right micrograph), and nuclear morphology examined after staining DNA with the Hoechst dye. The right-hand micrograph is taken at a 2.5-fold higher magnification than that in the centre. (**B**) Control: sperm heads were added at zero time to the same extract, but to which no HM fraction was added. This extract does not become apoptotic and readily supports assembly of meiotic spindles upon addition of CSF extract. Left micrograph: nuclear morphology after 45 min. Right micrograph: 0.5 vol of CSF extract was added at 90 min: 30 min later, metaphase spindles were assembled, as shown by incorporation of rhodamine-labelled tubulin in the spindle microtubules (right micrograph). Bars in (A), left micrograph, 5 μm; right micrograph, 2 μm.

As we were interested in the link that might exist between the release of oocytes from cell cycle arrest and the triggering of apoptosis, we investigated in the following experiments the effect of adding the catalytically inactive X-PAK1 mutant into Murray extracts.

While control extracts did not enter apoptosis, even after a 4 h incubation at room temperature, they readily underwent an apoptosis (Figure 11) indistinguishable from that caused by addition of mitochondrial fraction in excess, when recombinant X-PAK1-Cter K/R was added (0.2 mg/ml). In both cases (compare Figures 10 and 11), CPP32 underwent proteolysis ~90 min after adding either the recombinant protein or the mitochondrial fraction, while sperm nuclei underwent fragmentation and formed characteristic apoptotic bodies. All these events were suppressed by Bcl-2 or the DEVD peptide (data not shown).

In contrast, the catalytically active X-PAK1-Cter protein kinase failed to induce apoptosis under the same conditions (Figure 11). As the X-PAK1-Cter K/R mutant was expressed in fusion with Mal-BP, Mal-BP alone was added as a further control. In contrast to X-PAK1-Cter K/R, Mal-BP neither induced CPP32 proteolysis nor any other event of apoptosis (not shown), even when used at a higher concentration.

We were concerned that induction of apoptotic events

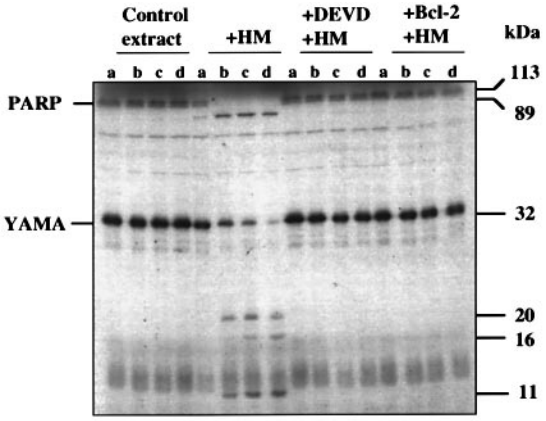

Fig. 10. Time course of PARP and YAMA/CPP32 cleavage in *Xenopus* egg extracts. [^{35}S]CPP32 and [^{35}S]PARP were generated by *in vitro* transcription/translation and added at the start of incubation to the extracts without (control) or simultaneously with 0.1 vol of a concentrated heavy membrane fraction (+HM) prepared from the same eggs, in the absence or presence of either the DEVD peptide inhibitor (10 nM) or Bcl-2 in a COS cell extract. Samples (3 μl) were removed at 60 (a), 90 (b), 150 (c) and 210 min (d) and analysed by SDS–PAGE and autoradiography. The molecular weights (kDa) indicated on the right were estimated from the position of marker proteins run on the same gel.

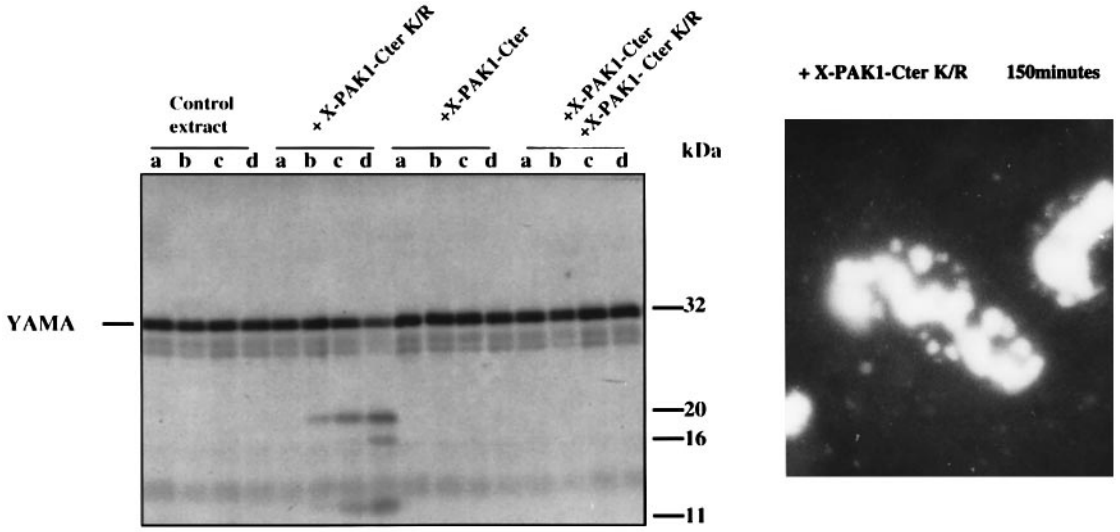

Fig. 11. Time course of YAMA/CCP32 cleavage in *Xenopus* egg extracts. [^{35}S]CPP32 was added at the start of incubation without (control) or simultaneously with the recombinant X-PAK1-Cter K/R mutant (0.2 mg/ml), the recombinant X-PAK1-Cter kinase (0.2 mg/ml) or both X-PAK1-Cter (0.2 mg/ml) and X-PAK1-Cter K/R (0.2 mg/ml).In this last case, extracts first received the X-PAK1-Cter kinase, and 30 min later (time 0) the kinase-dead mutant was added, simultaneously with [^{35}S]CPP32. Samples were removed at 60 (a), 90 (b), 150 (c) and 210 min (d) after [^{35}S]CPP32 addition and analysed by SDS–PAGE and autoradiography. The micrograph on the right shows the characteristic apoptotic bodies originating from sperm nuclei left for 120 min in egg extract containing the X-PAK1-Cter K/R mutant.

by the kinase-dead mutant could still have been an 'unspecific' effect due to addition of a possibly misfolded recombinant protein. This hypothesis was difficult to evaluate, as the K/R mutation that suppressed protein kinase activity also suppressed an easy way to check the protein for correct folding. We reasoned that, if the catalytically inactive X-PAK1-Cter mutant acts 'specifically', it should do so by competing with some *Xenopus* homologue of the Ste20/PAK family of protein kinases (possibly X-PAK1 itself) for some unknown component in the egg extract. This led us to investigate whether the catalytically active X-PAK1-Cter recombinant protein could suppress induction of apoptosis by its catalytically inactive counterpart.

As shown in Figure 11, this was indeed found to be the case, as prior addition of the catalytically active protein prevented CPP32 proteolysis upon addition of the kinase-dead mutant protein. Rescue was not observed on prior addition of Mal-BP or a Mal-BP fusion protein encoding the N-terminal domain of X-PAK1 (not shown).

Taken together, the above experiments demonstrate that apoptosis can be triggered by at least two procedures in *Xenopus* eggs extracts. The first one consists of adding a mitochondrial fraction in excess. The second consists of adding a kinase-dead mutant of X-PAK1 deleted of its N-terminal domain. To investigate whether mitochondria were still required for apoptosis to occur in the second procedure, the control extracts were further centrifuged at 100 000 *g* for 1 h, after which the cytosolic supernatant and light-membranes layer was recovered and mixed, while the mitochondrial pellet was removed. When X-PAK1-Cter K/R was added to the reconstituted extract free of mitochondria, apoptosis did not occur (Figure 12). Further addition of the mitochondrial pellet to its usual concentration in the extract restored responsiveness to X-PAK1-Cter K/R (Figure 12), and this was suppressed by Bcl-2 (Figure 12). No cleavage of CPP32 was detected in the

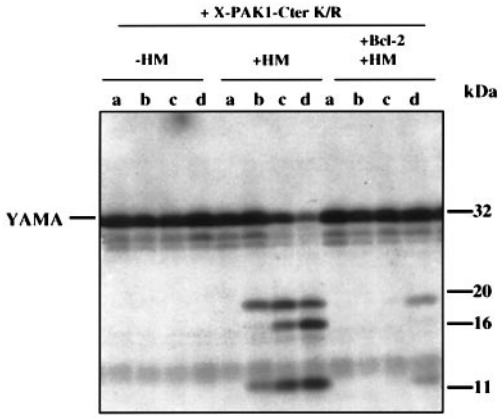

Fig. 12. Time course of CPP32 cleavage following addition of X-PAK1-Cter K/R to *Xenopus* egg extracts containing mitochondria (+HM) or not (–HM). [^{35}S]CPP32 was added at the start of incubation, simultaneously with X-PAK1-Cter K/R, into a reconstituted extract free of mitochondria (–HM: cytosolic 10^5 *g* supernatant + the light membranes) or a reconstituted extract with mitochondria (+HM: cytosolic 10^5 *g* supernatant + light and heavy membranes) in the absence (+HM) or presence (+Bcl2 + HM) of a COS lysate containing Bcl2. Samples were removed at 60, 90, 150 and 210 min after [^{35}S]CPP32 addition and analysed by SDS–PAGE and autoradiography.

extract reconstituted with mitochondria if X-PAK1-Cter K/R was not added (not shown).

In another set of experiments, the crude extract was incubated for 90 min with the dead-kinase mutant, before further fractionation at 250 000 *g*. The resulting cytosolic fraction (devoid of any membrane) was recovered and tested for its capacity to induce CCP32 cleavage and apoptosis in freshly prepared nuclei, assembled from sperm chromatin in 100 000 *g* cytosol containing light membranes but no mitochondria. CCP32 cleavage was indeed observed in the next 10 min following the addition of the primed ultra-high-speed supernatant, and nuclear

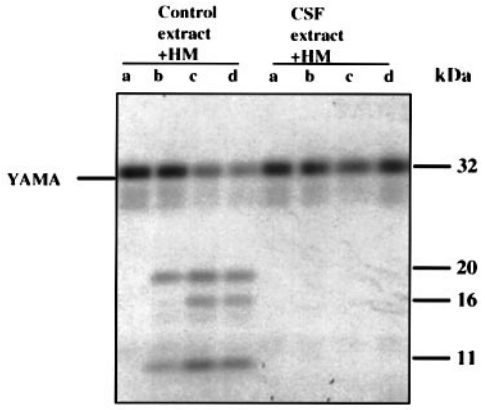

Fig. 13. Failure of CSF extracts to cleave CPP32 after addition of a heavy membrane mitochondrial fraction. A CSF extract was prepared from unfertilized eggs in the presence of EGTA to prevent crushing triggering parthenogenetic activation. A control extract was prepared from the same eggs in the absence of EGTA. Both extracts received at zero time 0.1 vol of a heavy membrane fraction (prepared in parallel from a control extract) and [^{35}S]CPP32. Samples were removed at 60 (a), 90 (b), 150 (c) and 210 min (d) after [^{35}S]CPP32 addition and processed for determination of CPP32 cleavage.

events of apoptosis readily occurred under such conditions (not shown).

We conclude that mitochondria are required for the catalytically inactive X-PAK1 mutant to trigger apoptosis in *Xenopus* egg extracts, but once this has occurred, mitochondria are no longer required to maintain apoptotic activity.

CSF extracts are less sensitive to apoptosis than interphase extracts

The extracts used in the above experiments were prepared from eggs that were crushed in the absence of a Ca^{2+} chelator, and thus entered interphase after cyclin degradation and MAPK inactivation (for review, see Lorca *et al*., 1994 and references therein). In the next experiments, 6 mM EGTA was included in the crushing buffer, to prevent cyclin degradation and MAPK inactivation. This 'CSF extract' (Masui and Markert, 1971) was processed as described in the above section and assayed for induction of apoptosis after increasing the ratio of the mitochondrial to the cytosol fraction, in parallel with an interphase extract. As shown in Figure 13, the interphase extract readily entered apoptosis, as shown by CPP32 proteolysis, when the amount of mitochondria was increased 2-fold by adding a concentrated suspension of purified mitochondria. In contrast, the CSF extract was resistant to this treatment. This was not due to the presence of EGTA in the CSF extract only, as addition of EGTA to the interphase extract before mitochondria neither prevented nor delayed CPP32 proteolysis.

Discussion

In this report, we have shown that microinjection of a catalytically inactive mutant of X-PAK1, a member of the *Xenopus* Ste20/PAK family of protein kinases, both facilitates release of oocytes from meiotic G_2 arrest and triggers apoptosis in cell-free extracts prepared from unfertilized eggs. Besides X-PAK1, this family contains at least five other members, and we have not yet investigated which specific role each of them plays in the fate of *Xenopus* oocytes. The link between release of meiotic G_2 arrest and apoptosis has a precedent, as ceramide, a reported inducer of apoptosis in many systems (Martin *et al*., 1995; Cuvillier *et al*., 1996; Verheij *et al*., 1996), was shown recently to induce meiotic maturation in *Xenopus* oocytes (De Smedt *et al*., 1995; Strum *et al*., 1995).

One of the most important results in the present work is that it is unnecessary to prime the toads with a specific hormonal regimen in order for them to lay eggs suitable for apoptotic extracts, in contrast to previous claims. For this reason, *Xenopus* egg extracts can be used to search for apoptosis-triggering signals. Any extract prepared from parthenogenetically activated eggs, that would otherwise faithfully reproduce *in vitro* cell cycle progression, will enter apoptosis, if mitochondria are added in excess to cytosol. This implies that eggs contain anti-apoptotic factors that neutralize the spontaneous tendency of mitochondria to release pro-apoptotic factors such as cytochrome c, that activates the cascade of caspases in cytosol (Liu *et al*., 1996; Kluck *et al*., 1997).

In the present work, we have been able to titrate one of these anti-apoptotic factors, using a dominant-negative strategy. This factor may regulate the activity of an anti-apoptotic member of the X-PAK/Ste20 family of protein kinases. Alternatively, it could be a substrate mediating its anti-apoptotic activity. Work is in progress to identify this factor and to analyse the cascade of events that links protein kinase activity of X-PAKs to the suppression of mitochondrial pro-apoptotic activity. Preliminary experiments suggest that several protein kinases, the activities of which change dramatically when extracts are induced to switch from cycling to apoptosis, are under the control of X-PAKs in *Xenopus* egg extracts. It is tempting to speculate that one of them may be involved in the post-translational control of Bcl-2 or of one of its cognates in *Xenopus* oocytes. For example, phosphorylation of death agonist BAD might prevent it from interacting with Bcl-2 or Bcl-xL homologues (Nuñez and Clarke, 1994; Reed, 1994), and free them to execute their anti-apoptotic function at the level of the outer mitochondrial membrane (Gajewski and Thompson, 1996; Wang *et al*., 1996; Zha *et al*., 1996). Ablation of Bcl-2 gene expression has indeed been shown to increase the number of oocytes undergoing apoptosis in the mouse gonad (Ratts *et al*., 1995), and *Xenopus* oocytes indeed express Bcl-2 related genes (Cruz-Reyes and Tata, 1995). Besides c-Raf (Wang *et al*., 1996) and c-Akt (Ahmed *et al*., 1997; Dudek *et al*., 1997; Kauffmann-Zeh *et al*., 1997; Kennedy *et al*., 1997) proto-oncogenes, X-PAK is thus the third serine/threonine kinase shown to deliver anti-apoptotic signals.

Both facilitation of progesterone-dependent release of oocytes from G_2 arrest and induction of apoptosis in cell-free extracts were observed using an inactive kinase mutant that was deleted of its 228-amino acid N-terminal half, located very close (22 amino acids) to the subdomain I of the catalytic domain, thus lacking the conserved domains responsible for binding of both Cdc42/Rac GTPases and SH3-containing proteins. In addition, microinjection into oocytes of a recombinant protein corresponding to the N-terminal half, or its addition to cell-free extracts, neither facilitated release from G_2 arrest nor

induced apoptosis. The dominant-negative effect of the X-PAK1 mutant was thus exerted either through its mutated catalytic domain or its short C-terminal tail.

Interestingly, it was shown recently that nearly all of the N-terminal non-catalytic half of Ste20 could be removed without affecting mating-specific responses in budding yeast (Peter *et al*., 1996; Leberer *et al*., 1997), indicating that molecular determinants which specify the pheromone signalling function of Ste20, including its regulation by the activated β- and γ-subunits of the mating response G-protein, must lie within either the kinase domain or the non-catalytic sequence C-terminal to the kinase domain. The mating response of budding yeast may be a paradigm for understanding how interaction of plasma membrane receptors with maturation-specific hormones (Kanatani and Hiramoto, 1970; Doree and Guerrier, 1975) is transduced into signals that control a major transition of the meiotic cell cycle in oocytes.

Heterotrimeric G-proteins have been reported to be involved in release of oocytes from G_2 arrest in both invertebrates and vertebrates. Microinjection of Gβγ into starfish oocytes mimic hormonal stimulation by 1-MeAde (Chiba *et al*., 1993). That of an antibody directed against Gαs mimics both progesterone and insulin in *Xenopus* oocytes and induces meiotic maturation in a protein synthesis-dependent manner. Our finding that a dominant-negative mutant of X-PAK1 deleted of its N-terminal domain facilitates induction of meiotic maturation by both progesterone and insulin suggests that an endogenous X-PAK homologue (not necessarily X-PAK1), regulated through interactions of its C-terminal domain by G-proteins, may contribute, besides protein kinase A (Maller and Krebs, 1977) to cell cycle arrest at the first meiotic G_2/prophase boundary. Such effect might occur by X-PAK inhibiting a step which is essential for meiotic maturation and common to the cascade of events triggered either by progesterone or insulin, as previously reported for protein kinase A (Matten *et al*., 1994).

In budding yeast, activation of Gβγ-dependent activation of Ste20 may also involve the adaptor protein Ste5, a scaffold protein which has been shown to interact with the kinases of the MAP kinase module (reviewed by Elion, 1995), to co-immunoprecipitate with Ste20 (Leeuw *et al*., 1995), and to associate with Gβ (Whiteway *et al*., 1995). A homologue of Ste5 has, however, not yet been described in higher eukaryotes.

Since this work was submitted for publication it has been shown—consistent with our results—that Jurkat cell lines expressing a dominant-negative PAK2 mutant had an enhanced externalization of phosphatidylserine at the cell surface in response to anti-Fas IgM (Rudel and Bokoch, 1997). Externalization of phosphatidylserine is an early and caspase-dependent event during apoptosis of cells from numerous lineages (Koopman *et al*., 1994; Martin *et al*., 1995, 1996; Vermes *et al*., 1995). Rudel and Bokoch (1997) used a catalytically inactive PAK mutant that, like our X-PAK1-Cter K/R mutant, was deficient in the ability to bind cdc42/Rac. Most likely, both mutants exert their dominant-negative effect by titrating some cellular component that interacts with their C-terminal domain. Both mammalian PAK2 and X-PAK1 contain in their C-terminal domain a stretch of conserved amino acids (SLTPYI, see Figure 1), also observed in yeast Ste20. Interestingly, the short tail C-terminal to the catalytic domain has been reported to be essential for Ste20 to respond to Gβγ (Leberer *et al*., 1997). Gβγ was also shown to stimulate an isotype of phosphoinositide 3-kinase, that positively controls activity of c-Akt, another anti-apoptotic serine/threonine kinase (Stoyanov *et al*., 1995).

In the present work, we have begun to unravel mechanisms which may possibly link in many species the rapid death by apoptosis of a majority of healthy oocytes, with the release after months or years from cell cycle arrest. Indeed, X-PAK1 or another member of the *Xenopus* Ste20/PAK family of protein kinases appears to be involved in both arrest of oocytes at G_2/prophase of the first meiotic prophase and prevention of apoptosis, although its mechanism of action remains to be elucidated. In contrast to the mechanisms of meiotic arrest and its release, the question of why oocytes of most vertebrate species arrest again at second meiotic metaphase has scarcely been addressed and has long remained a mystery. Recent studies on *Xenopus* and mice have suggested that metaphase arrest may occur to prevent inappropriate DNA replication prior to fertilization (Colledge, 1994; Furuno *et al*., 1994; Hashimoto *et al*., 1994; Sagata, 1996).

The present results suggest an alternative interpretation. Indeed, we found that extracts prepared from metaphase II-arrested eggs, in contrast to those prepared from parthenogenetically activated eggs, fail to enter apoptosis when mitochondria are added in excess to cytosol. Moreover, fully mature oocytes that do not undergo fertilization rapidly die by apoptosis in species such as starfish that do not arrest at metaphase II (A.Picard, personal communication). Finally, the most obvious consequence of disrupting *c-mos* in mouse oocytes, besides suppression of metaphase II arrest, is a high incidence of so-called 'cytoplasmic fragmentation' (Colledge, 1994), also observed in oocytes late in the apoptotic process (see Figure 2C in Tilly *et al*., 1997) and possibly similar to fragmentation of apoptotic cells before phagocytosis by neighbouring cells or nearby macrophages (Kerr *et al*., 1972). Hence, mechanisms for metaphase arrest may also have been selected during evolution because they protect oocytes from premature apoptosis before fertilization.

Materials and methods

cDNA cloning of X-PAK1

Total mRNAs from metaphase II-arrested eggs were prepared according to the method developed by Melton and Cortese (1979). Single-strand cDNAs were synthesized by reverse transcription, using a First Strand cDNAs Synthesis Kit (Pharmacia) and used as a template in PCRs. Degenerated oligonucleotides derived from the peptides sequences V A I K/Q M N (primer 1: 5′gtxgcxathaarmaratgaa3′) and E M I/V E G E (primer 2: 5′ggytcxccytcdaycatytcc3′) were chosen in the best conserved region of the kinase domains of Shk1 (Marcus *et al*., 1995), Ste20 (Leberer *et al*., 1992) and PAK65 (Manser *et al*., 1994). The amplified PCR product (480 bp) was subcloned in pBlue-Script vector (Stratagene). Approximately 20 independent clones were sequenced by T3 and T7 primers using a T7 sequenase kit (USB). Three clones, X1, X2 and X3 with different nucleotide sequences but sharing a strong homology to the kinase domain of Ste20 were chosen and used as probes to screen a λgt10 library of stage 6 oocytes (Dr D.A.Melton). 5×10^5 plaques were transferred to duplicate filters and hybridized at 50°C (in 6× SSC, 0.1% SDS, 5× Denhardt, 100 μg/ml of salmon sperm DNA), to the ^{32}P-labelled random primed PCR inserts of either X1, X2 or X3. Filters were washed (in 2× SSC, 0.1% SDS) at 37°C and 50°C, for 2 h. Three

rounds of plaque purification were performed. The partial X1 PCR probe yielded four positive plaques. Purification of λ phages from master plate infection, and of λ double-strand DNA were performed according to standard cloning protocols. *Eco*RI digests of λ DNA were cloned in pBlue-Script KS2. The sequences of the four clones revealed identical overlaps sequences. One of them had a complete open reading frame of 1572 bp, encoding for a putative 524-amino acids protein and a 3′ untranslated region bearing a AATAAAA poly(A) signal. This clone was named PBKS/X-PAK1.

Mal-BP constructs

Oligonucleotides XPAK1N5′ (ggcccggatcctccaacaatgctttggtgattg) and XPAK1N3′ (ggcccgtcgactcatcatgtcttgtctacgctgc); XPAK1C5′ (ggcccgaattcacagacgaggagatcttagag) and XPAK1C3′ (cccggcaagcttgtgctcctgtgacttcactcag), were used to amplify the N-terminal and C-terminal domains of X-PAK1 respectively. PCR products were subcloned into *Bam*HI and *Sal*I (for the N-terminal domain), *Eco*RI and *Hin*dIII (for the C-terminal domain), cloning sites of pMal-p2 (Biolabs) prokaryotic expression vector. The resulting plasmids were named pMal/X-PAK1-Cter and pMal/X-PAK1-Nter. The Mal-BP–XPAK fusion proteins were expressed and purified according to the manufacturer's recommendations (Biolabs).

Site-directed mutagenesis

Competent *Escherichia coli* CJ236 (dut ung) cells were transformed by PBKS/X-PAK1 and used to prepare single-strand DNA with R408 helper phage, according to the manufacturer's (Stratagene) protocol. The oligonucleotide X-PAK1K/R (5′gctgcaaattcatctgtctaatcgccacctcttgg3′) was used for site-directed mutagenesis of the lysine residue 279, located in the putative ATP binding site of the kinase domain of X-PAK1, using the protocol developed by Kunkel *et al*. (1987). A mutant clone (verified by sequence analysis) was further used to reconstruct a pMal/X-PAK1-Cter K/R clone.

Xenopus egg extracts, apoptotic assays

Crude *Xenopus* egg extracts were prepared according to a modification (Morin *et al*., 1994) of Murray's procedure (Murray, 1991). 100 000 *g* extracts were prepared by further fractionation, for 1 h, of the crude extract at 29 000 r.p.m. in the TST60 rotor of a Beckman centrifuge. Cytosolic and light membrane fractions were mixed, leading to the 100 000 *g* extract. The tan-coloured heavy membrane fraction was recovered separately, diluted in a 10-fold excess of ELB buffer (250 mM sucrose, 2.5 mM $MgCl_2$, 50 mMKCl, 1 mM DTT, 10 mM HEPES, pH 7.7), layered over lysis buffer plus 0.5 M sucrose and centrifuged at 22 000 r.p.m. for 20 min at 4°C in a TST60 rotor. The membrane pellet was resuspended, aliquoted, and frozen in liquid nitrogen. The heavy membrane fraction was added at a ratio of 1 to 10 to a fresh crude extract in order to induce the apoptotic process.

The tetrapeptide aldehyde AcNH2-DEVD-CHO (synthesized by SYNTEM, Nimes, France) was used at a final concentration of 10 nM. Typically, 1 μg of human Bcl-2, cloned in pRC-CMV (a kind gift of Dr U.Hibner), was used to transfect a 100 mm plate of Cos-7 cells at 40% confluency, using lipofectamine transfection protocol (Gibco, Bethesda Research laboratory), in accordance with the manufacturer's recommendations. At 48 h post-transfection, cells were harvested and washed twice in PBS buffer. Cell pellets were resuspended in an equal volume of 2% CHAPS, 20 mM HEPES, pH 7.4, 1 mM EDTA, 30 mM KCl, 20 μg/ml aprotinin/leupeptin, 1 mM DTT and left on ice for 15 min. Lysed cells were centrifuged at 13 000 r.p.m. for 5 min, supernatants were aliquoted and frozen in liquid nitrogen. Bcl-2 transfected extracts were used at a ratio of 1 μl per 100 μl of extract. Mock-transfected extracts added to *Xenopus* extracts did not delay the occurrence of apoptosis.

Bcl-2-transfected extracts and DEVD-CHO were incubated for 30 min with the *Xenopus* egg extract, before addition of the heavy membrane fraction or the purified recombinant truncated X-PAK1 (2 μl, at 0.2 μg/μl, per 50 μl of extract), which corresponds to time 0 in all experiments. At time 0, the apoptotic markers YAMA and PARP, ^{35}S-labelled and *in vitro*-translated were also added at a ratio of 2 μl per 50 μl of extract. *In vitro* translation of pcDNA3 YAMA and PARP were performed using the TNT coupled reticulocyte lysate kit (Promega), according to the manufacturer's instructions.

Nuclei, assembled from demembranated sperm nuclei (Lohka and Masui, 1984) were observed with a fluorescent microscope using Hoechst 33342 to stain chromosomes. Spindles were assembled as described previously (Morin *et al*., 1994).

Overlay assays and in-gel kinase assays

5 μg of purified recombinant Mal-BP alone or in fusion with X-PAK1-Cter or X-PAK1-Nter were loaded onto a 12% SDS–polyacrylamide gel. After electrophoresis the gel was transferred to PVDF membrane. The membrane was incubated in PBS, 1% BSA, 0.1% Triton X-100 and 5 mM DTT. Purified recombinant proteins GST–Rac1 and GST–Cdc42 were expressed and purified as described by Manser *et al*. (1992). 10 μg of purified recombinant GTPase was incubated with 2 μM [γ-^{35}S]GTP (in 25 mM MES, pH 6.5, 50 mM NaCl, 5 mM EDTA, 0.05% Triton X-100) for 30 min at room temperature, before dilution in 10 ml of binding buffer (25 mM MES, pH 6.5, 0.5 mM GTP, 5 mM $MgCl_2$, 50 mM NaCl, 5 mM DTT). Incubation with the PVDF membrane was performed for 15 min at room temperature (Martin *et al*., 1995). The blot was washed several times (in 25 mM MES, pH 6.5, 5 mM $MgCl_2$, 0.05% Triton X-100). Hyperfilm was exposed to the filter for 48 h.

In order to test the kinase activity of X-PAK1-Cter or its mutant, 2 or 3 μl of extract containing the recombinant proteins was loaded on 15% polyacrylamide SDS gels polymerized in the presence or absence of the MBP substrate. Gels were processed as described by Shibuya *et al*. (1992).

Embryos and Western blot analysis

Sexually mature adult *Xenopus laevis* were obtained from the CNRS local farm. Females were induced to ovulate by injection of 500 units of HCG (human chorionic gonadotropin). Eggs were fertilized artificially and embryos dejellied in 2% cysteine (pH 7.8) as described by Newport and Kirschner (1982). Embryos were cultured in MMR/4. Pools of several embryos were homogenized in lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 2 mM DTT, 10 μg/ml aprotinin/leupeptin). Lysates were centrifuged at 13 000 r.p.m. for 10 min at 4°C. Supernatants were recovered and protein concentrations estimated by spectrophotometry. 150 μg of total protein were separated by 10% SDS–PAGE and immunoblotted with purified polyclonal antibodies developed in rabbit against the C-terminal peptide of X-PAK1 at a 1/1000 dilution. Binding was visualized by a secondary antibody linked to horseradish peroxidase and chemiluminescence (ECL, Amersham).

Xenopus oocytes and microinjection

Females were injected with 100 units pregnant mare's serum gonadotropin (PMSG, Sigma), 48 h before oocyte removal. Females were anaesthetized with MS222 and fragments of ovary removed through a small incision in the abdominal wall. Stage 6 oocytes were prepared using 2.0 mg/ml collagenase to release the oocytes from the ovarian follicles. Oocytes were washed in OR2 (Unkeless *et al*., 1985). Typically, 40 nl of recombinant protein at 0.5 μg/μl was microinjected per oocyte.

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