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Involvement of K-Ras during meiotic division in *Xenopus laevis* oocytes

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Ras proteins, molecular switches in numerous biological pathways, are known to participate in the physiological mitosis cycle of somatic eukaryotic cells, but have not been indicated as components of physiological pathways during meiotic division cycles. Here we investigate involvement of Ras during meiotic maturation of *Xenopus laevis* oocytes using an endogenous *X. laevis* system. Purified Xe K-Ras 2B L61/S183 and Xe H-Ras V12 mutant and corresponding wild type proteins were microinjected into stage VI prophase oocytes, and the kinetic time course of maturation and activation of MAP kinase were monitored. Here we showed a potential inhibitory effect of K-Ras 2B L61/S183 on progesterone-induced meiotic maturation, suggesting that Ras protein or its downstream effectors are involved during meiosis in the oocyte. We also showed inhibition of H-Ras V12 effects by K-Ras 2B L61/S183 in a concentration-dependent mode, indicating that common effectors interact with different isoforms of Ras proteins in *X. laevis* oocyte.

Key words: *Xenopus*, Ras, MAPK, meiotic maturation

INTRODUCTION

Ras GTPases, such a H-Ras, K-Ras, N-Ras and others, operate as molecular switches in the signal transduction cascades controlling cell proliferation, differentiation and apoptosis. The biological activity of Ras proteins is regulated by the GTP/GDP cycle. Upstream regulators GEFs (guanine nucleotide exchange factors) induce the GDP-bound Ras transition to a GTP-bound form, whereas GTPase activating proteins (GAPs) enhance the intrinsic GTPase activity of Ras and restore Ras to its GDP-bound form [1]. Identification of Ras mutations in approximately 30% of all human tumors [2] illustrates the importance of Ras in cell biology and clinical research. The mutations are most frequent at codons 12, 13 and 61 that lead to inactivation of the GTPase activity and/or its sensitivity to GAP proteins, resulting in a constitutively GTP-bound Ras form [3].

Ras is posttranslationally modified in C terminus and targeted to the plasma membrane or endomembrane [3–5]. Mutation in the conserved CAAX motif blocks these posttranslational modifications and prevents Ras from binding to the membrane, thus disturbing its functioning [1, 6]. All the Ras proteins share a high homology in their N termini, but diverge in the C terminus. The differences in this hypervariable region are thought to be associated with different functions of proteins [3, 4, 6], which could be responsible for the specific Ras isoforms found mutated in different tumor types [3, 7]. K-Ras is mutated in pancreatic cancer (80–90%), lung cancer (30–60%) and colon cancer (30–50%). The N-Ras is implicated in melanoma (13%), liver cancer (30%) and acute myelogenous leukemia (30%). *H-Ras* gene is mutated in bladder cancer (10%) and kidney cancer (10%) [8, 2]. However, the precise nature of differences among Ras isoforms remains largely unclear [3, 4] as are also the roles and coeffectors of Ras in different cell types.

The role of Ras proteins in mitosis is well established, and it is known that in somatic cells Ras stimulates a multitude of downstream signaling cascades, and the main signaling cascade is Raf-MEK-MAPK [9–11]. The role of Ras in germinal cells, however, has not been investigated so intensively and remains to be clarified.

The female germinal cell, the oocyte, is a cell where all molecules necessary for the further embryonic development that occurs after fertilization are accumulated. Thus, the oocytes are a more complete system for signal transduction investigations, where the Ras proteins can be investigated in more detail, and new effectors and interactions could be

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determined. An example is the Mos protein kinase, which is specifically expressed and functions during meiotic maturation (or G2/M progression) of vertebrate oocytes [12–15].

Xenopus laevis oocytes are the most popular and useful model system for studying signaling pathways. They are naturally arrested in the prophase of the first meiotic division. This block is released by progesterone, triggering the activation of MPF (M-phase Promoting Factor) and phosphorilation of MAPK. Then the oocyte completes the first meiotic division and arrests in the metaphase of the second meiotic cell division, a process known as meiotic maturation or GVBD (Germinal Vesicle Breakdown) [16]. Here MAPK kinase activation mostly depends on Mos, and the early process of Mos activation remains unclear [17–19]. Recently it has been shown that injection of Xe H-Ras V12 mutant activates MAPK by two pathways, one being independent of protein synthesis and the other one requiring Mos synthesis to allow a full activation of MAPK [20]. Injection of Xe H-Ras N17, a constantly GDP-bound mutant, totally inhibits Xe H-Ras V12 effects, but cannot inhibit Pg-induced meiotic maturation [20]. In the absence of external stimuli, in a cell there still can be a small amount of GTP-bound Ras proteins, and Xe H-Ras N17 preventing the endogenous GDP-bound Ras to be switched to the GTP form cannot block theirs signal.

Here we used another type of interfering mutant – the Xe K-Ras 2B L61/S183 cytosolic GTP-bound protein, which fails to bind membrane and sequesters effectors to the cytosol thus preventing endogenous Ras to interact with them and target them to the membrane. Despite their potential utility, cytosolic GTP-bound Ras proteins are not well characterized [21], and the Ras mutant that blocks signal cascade at the effectors' level could be more informative than a Ras N17-type mutant.

MATERIALS AND METHODS

Materials. *Xenopus laevis* adult females (CNRS, Rennes, France) were bred and maintained under laboratory conditions.

Mutagenesis of *Xenopus laevis H-Ras* and *K-Ras 2B* genes

Xenopus laevis H-ras was cloned as described [20]. Cloned *H-ras* coding sequence cDNA and *Xenopus laevis K-ras* coding sequence cDNA (a kind gift of Dr. Y. Andeol) were recloned in a pUH 25-2 bacterial expression vector [22]. Site-directed mutagenesis was performed using proofreading DNA polymerase *Pfu* (Fermentas). Sequence was confirmed by sequencing (ABI Prism 377, Applied Biosystem).

Purification of bacterially expressed recombinant proteins

Expression of proteins in ER2267 *E. coli* strain was induced by 0.5 mM of isopropyl-β-D-thiogalactopyrano-side for 3 h. Bacteria were lysed by sonication in 50 mM Tris, pH 7.3, 0.1 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 5 mM benzamidine, 1 mg/ml lysosyme, 1% NP 40, 1% Triton X-100 and 0.6% CHAPS. The lysate was centrifuged for 1 h at 100 000 g, the supernatant was collected and dialyzed overnight against buffer A (20 mM Tris pH 8.0,1 mM EDTA, 5 mM $MgCl_2$, 1 mM β-mercaptoethanol) and then applied on a 15 ml FFQ anion-exchange column (Applied Biosystem) equilibrated in buffer A. After washing with buffer A, proteins were eluted with a 230 ml gradient from 0 to 0.25 M NaCl in buffer A. Fractions were analyzed on 15% SDS PAGE [22]. The fractions containing the recombinant protein were concentrated against dry polyethylene glycol (20000 kDa) and loaded on a Superose 12 gel filtration column (Applied Biosystem) equilibrated in buffer A supplemented with 0.1 M NaCl. Fractions containing the recombinant protein were concentrated against dry polyethylene glycol and the aliquots were frozen at –80 °C.

Xenopus oocytes treatments

An adult *Xenopus* female was anaesthetized in MS222 (Tricaine methane sulfonate solution 1–2 g/l buffered using 0.5–1 g/l $NaHCO_3$) for 10–20 min. Isolated oocytes were treated with 40 mg/100 ml of dispase for 3–4 h and with 50 mg/100 ml collagenase for 1 h, then washed with 2 l of Merriam (8.8 mM NaCl, 0.033 mM $Ca(NO_3)_2$, 0.1 mM KCl, 0.041 mM $CaCl_2$, 0.082 mM $MgSO_4$, 1 mM Hepes). Stage VI oocytes were collected and microinjected with various Xe H-Ras V12 and Xe K-Ras 2B L61/S183 concentrations. The oocytes were also incubated in the presence of 1 μM progesterone one or more hours after injection. Oocyte maturation was monitored following the appearance of a white spot at the animal pole.

Xenopus oocyte extracts

Matured and non-matured oocytes were collected and homogenized at 4 °C in 5 volumes of EB (80 mM β-glycerophosphate, pH 7.3, 20 mM EGTA, 15 mM $MgCl_2$, 1 mM dithiothreitol) with protease inhibitor (1 mM Pefablock). The lysates were centrifuged at 15000 g at 4 °C for 15 min the supernatants were collected and analyzed.

Western blotting

Samples (equivalent to 2 oocytes) were electrophoresed on 12.5% Anderson SDS-PAGE [24] and transferred to nitrocellulose membranes (Amersham). The rabbit polyclonal anti-MAPK was used (Santa Cruz Biotechnology, Inc.). The secondary HRP-conjugated antibodies (Jackson ImmunoResearch) were directed against the rabbit Ig and detected by chemiluminescense (NEN).

RESULTS AND DISCUSSION

K-Ras did not induce meiotic maturation in *X. laevis* oocytes. Previously we have reported induction of GVBD by injection of Xe H-Ras in *X. laevis* oocytes [20]. To test if different Ras isoforms play different roles in the oocyte, here we examined K-Ras using an endogenous *Xenopus laevis* system.

Recombinant Xe K-Ras 2B (wild type and GTP-bound V12 mutant) protein was produced and purified as described in Materials and Methods and was injected in the oocyte.

Injection of either wild type and GTP-bound mutant of K-Ras did not induce GVBD opposite the injection of the H-Ras isoform [20], indicating different roles of Ras isoforms in the oocyte.

Interfering K-Ras mutant inhibits progesterone-induced maturation in *X. laevis* oocytes. To determine the importance of K-Ras for oocyte meiotic maturation we used a cytosolic GTP-bound K-Ras mutant. Stage VI prophase oocytes were injected with Xe K-Ras 2B L61/S183 or/and incubated with progesterone (Pg). As expected, Xe K-Ras 2B mutant didn't induce meiotic maturation and didn't activate MAP kinase. Instead, after 3–4 h independently of progesterone we observed pigment rearrangements in the animal pole (Fig. 1 bottom), which was specific to this mutant. The maturation of oocytes reached only 20–30% and MAPK activation wasn't complete (Fig. 1 top). Oocytes after injection didn't survive for a long time compared with control prophase oocytes. These results show the potential capability of Xe K-Ras 2B L61\S183 to inhibit progesterone signaling and imply an idea, that Xe K-Ras 2B interacts with some effectors that are important for the oocytes' vitality. The failure to observe inhibition of progesterone-induced meiotic maturation when a GDP-bound interfering Ras mutant was used [20] suggests the presence of GTP-bound Ras proteins in G2 arrested oocytes, which are not affected by the GDP-bound Ras mutant.

Insufficient inhibition can also be dependent upon progesterone addition time. Here we added Pg 1 h after injection, but it could be still insufficient for Ras to sequester all effectors. To examine

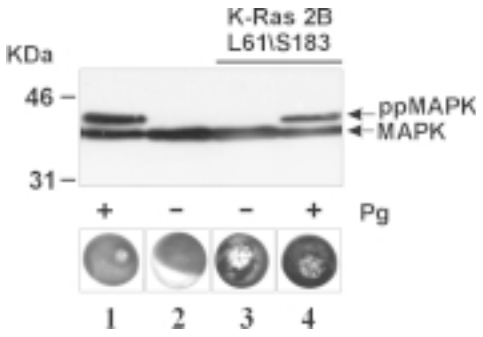

Fig. 1. Western analysis and pigment rearrangements of oocytes injected with Xe K-Ras 2B L61/S183 and/or incubation with progesterone.
A. Imunoblotting with anti-MAPK antibodies. (ppMAPK – phosphorilated MAPK form)
1. Oocytes treated with 10 μM progesterone
2. Prophase I oocytes
3. Oocytes injected with 75 ng of K-Ras 2B L61/S183 protein
4. Oocytes injected with 75 ng of K-Ras 2B L61/S183 and treated with progesterone 1 hour after injection.
B. Morphological changes of oocytes.

a correlation between the K-Ras inhibitory effect and progesterone addition time, in parallel we added progesterone 2 and 3 h later after injection. We saw that oocytes injected with Xe K-Ras 2B L61/S183 with a 2-h delay reached only 12% and after a 3-h delay only 8% (Fig. 2). These results suggest that G2-arrested oocytes may have some Ras effectors and to sequester them the observed lag period is required.

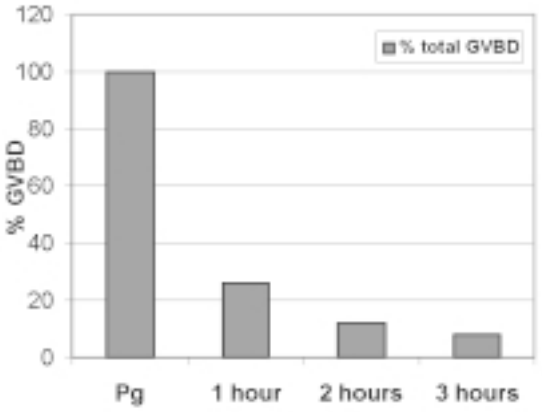

Fig. 2. Dependence of Xe K-Ras 2B L61/S183 inhibitory effect on progesterone addition time
1. Progesterone-induced total GVBD
2. Total % of GVBD after K-Ras 2B L61/S183 injection. Progesterone was added 1 hour after injection
3. Total % of GVBD after K-Ras 2B L61/S183 injection. Progesterone was added 2 hours after injection
4. Total % of GVBD after K-Ras 2B L61/S183 injection. Progesterone was added 3 hours after injection

Inhibition of H-Ras-induced meiotic maturation by interfering K-Ras mutant. To investigate whether Xe K-Ras 2B transfers signal through the same effectors as do other Ras proteins and whether it is able to inhibit them, we investigated its inhibitory effect on the H-Ras V12 protein, injecting both proteins in one oocyte and also incubating them with Pg. Previously [20] it has been shown that Xe H-Ras V12 induces GVBD alone and accelerates Pg-induced maturation. In the absence of Pg, oocytes injected with both mutant proteins didn't mature and after 8 h there was no activation of MAPK, showing that Xe K-Ras 2B interfered with Xe H-Ras V12 for effectors and inhibited its effects (Fig. 3 A). In the presence of progesterone the oocytes injected with both mutants matured more slowly than the oocytes injected with Xe H-Ras V12 in the presence of Pg (Fig. 3 B), suggesting that progesterone restores the MAPK pathway through an alternative way. To test whether the inhibitory effect depends on the protein concentration ratio, oocytes were injected with a double amount of K-Ras 2B L61/S183 together with one portion of H-Ras and also treated with progesterone. We didn't observe GVBD, but pigment rearrangement as in oocytes injected with K-Ras 2B alone and their vitality were very low. Addition of progesterone didn't restore GVBD and the activation of MAPK. However, after an overnight incubation we found some activated MAPK (Fig. 4). These results suggest that the inhibitory effect of K-Ras 2B L61/S183 depends on the concentration ratio and the vitality of oocytes depends on the K-Ras 2B L61/S183 concentration, and neither H-Ras V12 nor progesterone can restore it.

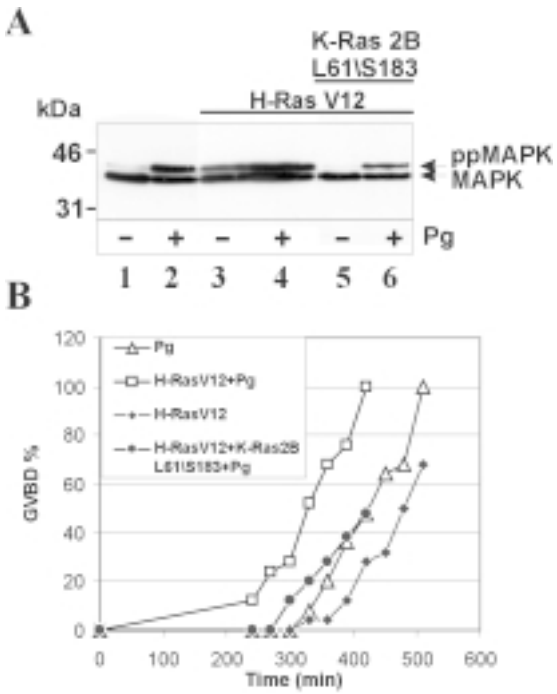

Fig. 3. Inhibitory effect of K-Ras 2B L61/S183 mutant.
A. Western analysis of oocytes injected with H-Ras V12 protein and/or with K-Ras 2B L61/S183 using anti-MAPK antibodies
1. Prophase oocytes
2. Oocytes treated with 1 μM progesterone
3. Oocytes injected with 75 ng H-Ras V12
4. Oocytes injected with 75 ng H-Ras V12 treated with 1 μM progesterone for 8 hours
5. Oocytes injected with 75 ng of H-Ras V12 and 75 ng of K-Ras 2B L61/S183
6. Oocytes injected with 75 ng of H-Ras V12 and 75 ng of K-Ras 2B L61/S183 treated with 1 μM progesterone.
B. GVBD time course

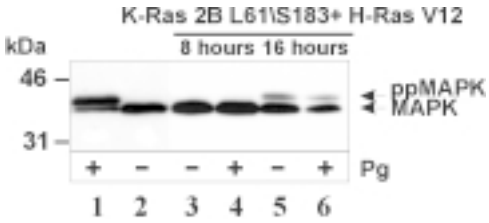

Fig. 4. K-Ras 2B L61/S183 inhibitory effect depends upon the concentration ratio of interfering and GTP form mutant proteins
1. Oocytes treated with 1 μM progesterone
2. Prophase oocytes
3. Oocytes injected with 150 ng of K-Ras 2B L61/S183 and 75 ng of H-Ras V12 after 8 hours
4. Oocytes injected with 150 ng of K-Ras 2B L61/S183 and 75 ng of H-Ras V12 and treated with progesterone for 8 hours
5. Oocytes injected with 150 ng of K-Ras 2B L61/S183 and 75 ng of H-Ras V12 after 16 hours
6. Oocytes injected with 150 ng of K-Ras 2B L61/S183 and 75 ng of H-Ras V12 and treated with progesterone for 16 hours.

Here we have shown that the K-Ras 2B L61/S183 protein is capable of inhibiting progesterone-induced maturation, suggesting that K-Ras or its downstream effectors participate in the physiological meiotic maturation in an oocyte. To fully sequester the effectors K-Ras2B needs more than 3 h. Oocytes injected with an interfering mutant show specific morphological changes and their vitality decreases, allowing a suggestion that K-Ras 2B interacts and sequesters some yet unidentified effectors important for oocyte survival. Inhibition of H-Ras effect by an interfering K-Ras mutant indicates that both Ras isoforms compete for the same effectors in the oocyte.

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K-RAS DALYVAVIMAS *Xenopus laevis* OVOCITŲ MEJOTINIAME DALIJIMESI

S a n t r a u k a

Xe K-Ras 2B interferuojančio mutanto savybės buvo tirtos endogeninėje *X. laevis* sistemoje. Išgryninti Xe K-Ras 2B L61/S183 ir Xe H-Ras V12 baltymai buvo sušvirkščiami į *X. laevis* VI stadijos profazinius ovocitus. Stebėta ovocitų mejotinio brendimo kinetika ir MAP kinazės aktyvacija. Paaiškėjo, kad Xe K-Ras 2B L61/S183 injekcija sumažina ovocitų gyvybingumą ir iš dalies inhibuoja progesterono paskatintą mejotinį brendimą, tačiau inhibicijos lygis priklauso nuo progesterono pridėjimo laiko. Taip pat nustatyta, kad Xe K-Ras 2B L61/S183 interferuojantis mutantas visiškai inhibuoja H-Ras V12 veikimą sušvirkštus abu baltymus kartu ir kad ši inhibicija priklauso nuo baltymų koncentracijos santykio.