Volume 234, number 2, 426–430 FEB 06103 July 1988

Oncogenic *ras* protein induces meiotic maturation of amphibian oocytes in the presence of protein synthesis inhibitors

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Received 26 May 1988

Microinjection of the activated *ras* oncogenic protein can induce the meiotic maturation of *Xenopus laevis* oocytes, a process that can also be triggered by progesterone or high concentrations of insulin. Cycloheximide and puromycin, well-known inhibitors of protein synthesis, block the maturation process induced by progesterone and insulin but do not affect the maturation caused by H-*ras*$^{lys\ 12}$ protein microinjection. Theophylline, an inhibitor of cAMP phosphodiesterase that also affects oocyte protein synthesis, does cause a partial inhibition of *ras* protein-induced maturation. These findings indicate that *ras* protein acts on the oocyte maturation process at a point that is downstream of the protein synthesis requirement, a characteristic shared with the maturation promoting factor, an activity that appears in oocytes and mitotic cells at the onset of cell division.

Maturation; Protein-synthesis inhibitor; Oocyte; *ras* protein

1. INTRODUCTION

Full-grown amphibian oocytes are arrested in the first meiotic prophase. Progesterone or high concentrations of insulin can induce these cells to progress to the second meiotic metaphase in a process that is known as meiotic maturation (review [1]).

This process is very complex and shares many of the features of mitotic cell division. Some of the most relevant molecular events that have been found to occur in oocytes during progesterone-induced maturation are summarized below.

(i) A transient decrease in cAMP is caused by the atypical inhibition of adenylate cyclase by progesterone (review [2]). Inhibition of this decrease by activators of the adenylate cyclase such as cholera toxin or by theophylline prevents progesterone-induced maturation. Activation of cAMP-dependent protein kinase blocks maturation while inhibitors of this activity induce the maturation process [3].

(ii) An increase (2–3-fold) in oocyte protein synthesis occurs 2 h after progesterone treatment and this is post-transcriptionally regulated [4]. Inhibitors of protein synthesis added up to 2 h after hormone treatment block maturation [5].

(iii) A maturation-promoting factor (MPF) is observed in the oocyte cytoplasm approx. 2–3 h after progesterone treatment. Microinjection of MPF causes rapid maturation of oocytes without exposure to hormone. The maturation caused by MPF cannot be blocked by protein synthesis inhibitors. This factor has not been completely purified but partially purified preparations contain protein kinase activity. MPF appears during mitosis of many different eukaryotic cells and is functionally active in oocytes [6,7].

(iv) After MPF microinjection or 3–4 h after progesterone treatment, there is a significant increase in oocyte protein phosphorylation [8]. Two proteins whose phosphorylation increases concomitantly with maturation have been identified as

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Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/88/$3.50

ribosomal protein S6 [9], and the nuclear nucleosome assembly factor, nucleoplasmin [10].

In animal cells, the family of *ras* genes produces closely related p21 proteins that bind GTP and GDP and have intrinsic GTPase activity. Specific mutations activate their malignant potential and the resulting oncogenes code for p21 proteins that have transforming activity (review [11]). The microinjection of the activated c-Harvey *ras* protein induces maturation in *Xenopus laevis* oocytes more efficiently than the normal c-Harvey *ras* protein [12].

Supporting evidence for the possible participation of the endogenous *ras* protein in oocyte maturation has been obtained by microinjection of monoclonal antibodies that inactivate *ras* proteins. The presence of these antibodies inhibits the induction of maturation by insulin but not that triggered by progesterone [13,14]. This has suggested that parallel pathways for meiotic maturation may be operative in the oocyte. Here, we demonstrate that the induction of oocyte maturation caused by microinjection of activated *ras* protein cannot be blocked by the inhibition of protein synthesis although insulin-caused maturation does require protein synthesis.

2. MATERIALS AND METHODS

Adult female *X. laevis* were obtained from Nasco (Fort Atkinson, WI) or the South African Animal Farm (Cape Town, South Africa). Both hormonally stimulated animals [7], which received by injection low levels of human chorionic gonadotropin (Sigma CG-10) and non-stimulated animals were used. Oocytes were removed surgically from animals anesthetized by hypothermia. Stage VI oocytes were hand-separated, into Barth solution (88 mM NaCl, 2.4 mM $NaHCO_3$, 1 mM KCl, 0.82 mM $MgSO_4$, 0.74 mM $CaCl_2$, 0.33 mM $Ca(NO_3)_2$, 10 mM Tris-HCl, pH 7.6, and 10 μg/ml each of penicillin and streptomycin sulfate) and used the same day. For some experiments, KCl was omitted from the saline solution. Removal of follicle cells by collagenase treatment (Sigma type 1A; 4 h at 1.5 mg collagenase/ml Barth solution, followed by extensive rinsing) did not alter the response of oocytes to *ras*-induced maturation.

Maturation was induced by the addition of 10 μM progesterone (Sigma), 1 μM insulin (Miles) or by *ras* protein microinjection, using groups of about 30 oocytes. Controls were performed with the corresponding vehicle. Maturation in the presence of inhibitors was measured (without preincubation of the oocytes) at 20 μM cycloheximide, 500 μM puromycin or 1 mM theophylline in Barth solution [5].

Maturation was scored, routinely after 8–10 h but occasionally at 18 h, by microscopic examination for the presence of the nucleus in the treated oocytes after fixation with 5% trichloroacetic acid. The absence of the nucleus indicated that 'germinal vesicle breakdown' (GVBD) had occurred. In the oocytes microinjected with activated *ras* protein, the white spot in the pigmented animal pole that typifies GVBD was often larger and upon extended incubation (16–24 h) the pigmentation in the animal pole became mottled. When widespread cytolysis of the oocytes was observed, these cells were excluded from the tally. This phenomenon was particularly frequent in the oocytes obtained from frogs that had been primed with injections of human chorionic gonadotropin.

H-$ras^{lys\ 12}$ protein was prepared and purified from *E. coli* transformed with the expression plasmid PJCL30 [15] as described [16]. Microinjections were performed by injecting 25–50-nl volumes of *ras* protein diluted to 0.5 or 1 mg/ml in 10 mM Hepes, pH 7.6, and 1 mM $MgCl_2$.

3. RESULTS

Meiotic maturation is standardly assayed by scoring the disappearance of the nuclear membrane, or GVBD in oocytes fixed with trichloroacetic acid. In table 1, it can be seen that the presence of 20 μM cycloheximide or 500 μM puromycin can block completely the GVBD induced by progesterone or insulin. These concentrations of the drugs in the medium cause more than 95% inhibition of protein synthesis in the oocytes [17]. In this table it is also shown that the presence of the same inhibitors does not significantly decrease the GVBD brought about by the microinjection of the activated H-$ras^{lys\ 12}$ protein. The 5 experiments presented are representative of more than 12 separate experiments that showed essentially the same results.

However, some comments are in order with respect to the meiotic maturation caused by *ras* p21 microinjection. The external morphology of the oocytes microinjected with the activated p21 is different from that of the oocytes treated with hormone, depending on the amount of the oncogenic protein injected and on the hormonal state of the animal from which the ovary is obtained. With low amounts (~10–20 ng) of activated p21, the appearance of the white maturation spot on the pigmented animal pole is very similar to that observed with hormonally induced maturation. With higher amounts of the activated *ras* protein (above 30 ng), the pigment of the oocyte becomes patchy and 2–3 h after GVBD the cells appear to undergo massive cytolysis. With the oocytes treated with progesterone, such morphological changes occur only 20 h or more after GVBD. The

Table 1

The effect of protein synthesis inhibitors on the induction of oocyte maturation (GVBD) by hormones and microinjection of activated H-*ras*$^{lys\ 12}$ protein

Expt	Maturation inducer	Inhibitor added	% oocytes undergoing GVBD	% inhibition
1	progesterone (10 μM)	-	100	
		cycloheximide	0	100
	insulin (1 μM)	-	100	
		cycloheximide	0	100
	H-*ras*$^{lys\ 12}$ protein (20 ng)	-	91	
		cycloheximide	95	0
2	progesterone (10 μM)	-	100	
		cycloheximide	0	100
	insulin (1 μM)	-	100	
		cycloheximide	0	100
	H-*ras*$^{lys\ 12}$ protein (20 ng)	-	86	
		cycloheximide	76	12
3	progesterone (10 μM)	-	100	
		cycloheximide	0	100
	insulin (1 μM)	-	100	
		cycloheximide	0	100
	H-*ras*$^{lys\ 12}$ protein (28 ng)	-	67	
		cycloheximide	55	18
4	progesterone (10 μM)	-	94	
		cycloheximide	0	100
	H-*ras*$^{lys\ 12}$ protein (20 ng)	-	70	
		cycloheximide	87	0
5	progesterone (10 μM)	-	100	
		puromycin	0	100
	H-*ras*$^{lys\ 12}$ protein (17 ng)	-	95	
		puromycin	81	15

Stage VI oocytes were manually separated from ovaries obtained from adult *X. laevis* previously treated with human chorionic gonadotropin [7] and were placed in Barth solution. Groups of 30–35 oocytes were incubated with progesterone or insulin and, where indicated, with the simultaneous addition of either 20 μM cycloheximide or 500 μM puromycin. Separate groups of oocytes were microinjected with 10–28 ng *ras* protein in 30–50-nl volumes and incubated either in Barth solution alone or with added inhibitor as given. Maturation was scored at 8–10 h and the absence of the nucleus confirmed by dissecting the oocytes fixed in 5% trichloroacetic acid. Each experiment was carried out with oocytes from a different animal

experiments presented in table 1 were performed with oocytes from frogs that had been 'primed' by recurrent injection of low doses (50–100 units) of human chorionic gonadotropin [7]. Cells from untreated frogs showed slower kinetics of maturation with microinjection of activated *ras* protein, requiring up to 10 h to complete GVBD. This phenomenon is also well-documented for progesterone induction of maturation.

The presence or absence of K^+, which affects the maturation response to metal ions [18], did not significantly change the induction of maturation by *ras* protein microinjection (not shown).

Theophylline at 1 mM is a well-known inhibitor of cAMP phosphodiesterase and has been shown to inhibit the maturation of oocytes induced by progesterone, human chorionic gonadotropin and lanthanum ion [5]. It was also shown that 1 mM theophylline caused an inhibition of 50–60% of protein synthesis. Table 2 shows that the presence of this drug does result in a partial inhibition of the GVBD caused by activated *ras* protein. In this case, it is important to point out that approximately half of the nuclear breakdowns scored corresponded to nuclei that had migrated to the membrane of the animal pole but had not suffered

Table 2

Effect of theophylline on the meiotic maturation of oocytes (GVBD) caused by hormones and microinjection of activated H-*ras*$^{lys\ 12}$ protein

Expt	Maturation inducer	Inhibitor added	% oocytes undergoing GVBD	% inhibition
1	progesterone	-	88	
		theophylline	0	100
	H-*ras*$^{lys\ 12}$ protein (10 ng)	-	47	
		theophylline	19[a]	59
2	progesterone	-	83	-
		theophylline	0	100
	H-*ras*$^{lys\ 12}$ protein (20 ng)	-	85	-
		theophylline	54[a]	36

[a] About half of the oocytes in these groups showed abnormal maturation and the % GVBD given includes those oocytes which had the typical white spot but in which nuclear material had migrated toward the animal pole and had a flattened funnel shape

Stage VI oocytes were manually separated from ovaries obtained from adult female *X. laevis* which had not been primed by previous hormone treatment. Incubations and microinjections were carried out as given in table 1. Theophylline was at 1 mM. Maturation was scored at 18 h

complete dispersion of their nuclear contents which were funnel-shaped. This result is similar to the observations of Birchmeier et al. [12] after treatment with cholera toxin of oocytes microinjected with *ras* p21.

4. DISCUSSION

The present results clearly demonstrate that the induction of oocyte meiotic maturation by microinjection of activated H-*ras*$^{lys\ 12}$ proteins cannot be blocked by inhibitors of protein synthesis. This finding is important because it provides some information regarding the place where *ras* may be operating in the cascade of events that lead to meiotic division. Fig.1 shows the possible sequence of events that may participate in oocyte meiotic maturation induced by the putative separate pathways of two hormones: progesterone and insulin. The progesterone pathway has been well documented [1] and some of its most salient features are discussed in section 1. It is clear that MPF appearance and increased specific protein phosphorylation are downstream of the protein synthesis requirement in that pathway [8].

Insulin maturation has been postulated to operate through a different and separate pathway and to include *ras* proteins on the basis of experiments whereby anti-*ras* monoclonal antibodies block insulin induction of maturation but not the process triggered by progesterone [13,14]. Pertinent to this relationship are the experiments of Stacey and co-workers [19] that demonstrated that *ras* antibodies block the transforming effects of oncogenes that, like insulin, involve membrane tyrosine kinase activities. The present work demonstrates that protein synthesis is required for insulin-induced maturation and that *ras* action in that pathway is downstream from the protein synthesis requirement. This is interesting because it separates *ras* from the primary membrane signal transduction mechanism for insulin where it has been generally postulated to act [11]. However, it is also clear that *ras* protein in the oocyte also binds to the membrane [12].

This observation also makes *ras* protein similar to MPF which appears to be a universal component of dividing cells. An important related observation is the recent finding that microinjection of activated *ras* protein into oocytes causes an increase in specific protein phosphorylation (Nebrada, A. et al., submitted).

The partial inhibition of activated *ras*-mediated oocyte maturation obtained with theophylline is not easily interpreted because this drug is known to have several effects. The capacity of theophylline to inhibit 50-60% of oocyte protein synthesis at the concentration used [5] cannot explain the decrease of *ras*-induced maturation because the more potent inhibitors of protein synthesis, cyclo-

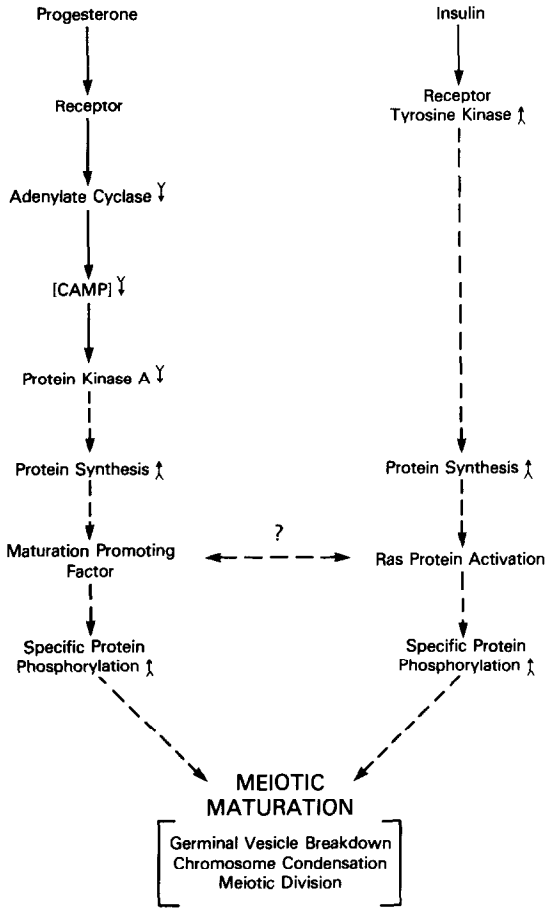

Fig.1. Possible alternative cascades for the induction of amphibian oocyte meiotic maturation by progesterone and insulin. Dashed arrows indicate unknown mechanisms that may connect different components of the cascade. This speculative scheme does not pretend to include all the information about oocyte meiotic maturation that is available.

heximide and puromycin, do not interfere with this process. Its effect as an inhibitor of cAMP phosphodiesterase which tends to increase intracellular cAMP could be related to the observed effect. This possibility is supported by the observation of Birchmeier et al. [12], who obtained similar partial inhibition and atypical nuclear behavior by treatment of *ras*-injected oocytes with cholera toxin, a protein which also causes an increase in intracellular cAMP through activation of adenylate cyclase.

Meiotic maturation of oocytes seems to be a valid model in which to study some of the factors that trigger cell division. It is very convenient that the oncogenic mutation that activates the transforming activity of *ras* proteins reflects itself in a highly increased capacity of these proteins to cause oocyte maturation, a process which can also be triggered by hormones and which can be studied in these cells in the absence of cell growth.

Acknowledgements: This work was supported in part by the National Council of Tobacco Research, FONDECYT-Chile, the OAS and the University of Chile. Part of this work was carried out while J.E.A. was a Fogarty Scholar-in-Residence at the NIH.

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