Oncogenic Ras triggers the activation of 42-kDa mitogen-activated protein kinase in extracts of quiescent Xenopus oocytes

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ABSTRACT Quiescent, full-grown Xenopus oocytes, which are arrested at the G2/M border of meiosis, contain an inactive 42-kDa mitogen-activated protein kinase (p42MAPK) that is activated when oocytes are stimulated to resume the meiotic cell cycle. We have made extracts from these oocytes that respond to four cell cycle activators: oncogenic [Val12]Ras proteins, clasm cyclins Aδ60 and Bδ97, and the phosphatase inhibitor okadaic acid. All four induce the tyrosine phosphorylation and activation of p42MAPK. Both cyclins and okadaic acid, but not [Val12]Ras, also lead to activation of the endogenous cyclin B/cdc2 kinase complexes in extracts of quiescent oocytes. Using extracts prepared from cycloheximide-arrested interphase cells, we show that although p42MAPK activation can occur in response to cyclin-activated cdc2, the Ras-induced activation of p42MAPK occurs without intervening cdc2 activation. Neither the nononcogenic [Gly12]Ras nor [Val12,Arg186]-Ras, a mutant that lacks the C-terminal consensus sequence directing prenylation and subsequent membrane association, is an effective activator of p42MAPK in vitro.

Ras proteins are low molecular weight, membrane-associated, guanine nucleotide-binding proteins involved in signal transduction events in a wide variety of organisms (1). Mutant, activated Ras proteins, with impaired ability to hydrolyze GTP, are commonly found in malignant transformed cells (2–7). Although Ras is known for the action of many other oncogene products and growth factors (8, 9), the function of Ras proteins in higher organisms is unclear. Virtually all mitogens and many other extracellular signals induce the rapid, transient activation of MAP kinases via phosphorylation on tyrosine and threonine residues (reviewed in refs. 10 and 11). The requirement for Ras in growth factor-induced activation of MAP kinases (8, 9, 12) and the ability of mutant, activated Ras to induce MAP kinase activation in cultured cells (8, 9, 13) suggest that Ras is a converging point for numerous signal transduction pathways. MAP kinase activation is perhaps the earliest known biochemical marker for Ras function in vertebrates.

Unlike most vertebrate somatic cells, which are arrested in G0, Xenopus oocytes are arrested in prophase at the G2/M border of meiosis. Progesterone stimulation leads to the activation of a preexisting 42-kDa MAP kinase (p42MAPK) (14–16) and cyclin B/cdc2 complexes known as pre-MPF (maturation-promoting factor; reviewed in ref. 17) and resumption of the cell cycle (reviewed in refs. 18 and 19). Since injection of oncogenic Ras into somatic cells or BrdUrc24 into oocytes can also lead to cell cycle reentry, oocytes offer a useful system in which these pathways can be analyzed. Here we show that injection of oncogenic Ras into G2/M-arrested oocytes leads to the activation of p42MAPK. We have also established a cell-free system from oocytes that maintains the quiescent state in vitro while retaining the capacity to respond to cell cycle activators. This cell-free system reproduces the Ras-dependent activation of p42MAPK, as well as other events that can lead to cell cycle reentry, including the cyclin-induced conversion of cyclin B/cdc2 pre-MPF complexes to active MPF.

MATERIALS AND METHODS

Construction of Escherichia coli Expression Vectors. PCRs (Taq polymerase, Perkin-Elmer) (25) were performed by Vincent Jung to generate Ha-ras genes with the desired mutations and BamHI as the cloning site (underlined). The 5' oligonucleotide used was 5'-CCCTGAGCATCATGAC-GAATATAAG-3' which created a BamHI site (underlined) upstream of the initiation codon (in bold); the 3' oligonucleotide was 5'-CTAGAGATCTCTAGAGACACACACCTT-3'. The 5' oligonucleotide used to mutate the membrane localization site was 5'-CTAGAGATCTCTAGAGACCAACGCCTT-3' with a point mutation (in bold) to change Cys186 (CAG) to Arg (CGC). The PCR templates were derived from [Gly12]Ha-ras (wild type) and [Val12]Ha-ras cDNAs (26). These PCR products were first cloned into ADH1 promoter-driven expression vectors for Saccharomyces cerevisiae (27) to confirm their biological activity (28). Expression of [Gly12]Ha-ras in yeast suppressed the temperature sensitivity of a strain containing a temperature-sensitive mutation ras224. Expression of [Val12]Ha-ras conferred heat shock sensitivity on a wild-type strain and suppressed the lethality of a strain containing cdc2533. Finally, expression of [Val12,Arg186]Ha-ras suppressed the heat shock sensitivity of a strain containing [Val12]RAS2 (29). The fragments were cloned into pTrcHis (Invitrogen, San Diego) and expressed as fusion proteins with N-terminal oligohistidine (30, 31).

Purification of Intact and Histidine-Tagged Fusion Proteins. Ha-Ras proteins were purified as described (32). His-tagged proteins were prepared according to ref. 31 and manufacturer's instructions (Invitrogen). The His-Ras proteins were solubilized in sonication buffer (10 mM Tris-HCl, pH 7.5/10 mM MgCl2/50 mM NaCl/1 μM phenylmethylsulfonyl fluoride/20 μM leupeptin/aprotinin (2 μg/ml)/2 μM pepstatin/0.5% (wt/vol) dodecyl β-p-maltoside (Boehringer Mannheim). The protein was bound to Pro-Bond resin (Invitrogen) overnight at 4°C and eluted with increasing imidazole concentrations. Fractions containing His-tagged proteins, identified by immuno blotting, were dialyzed against microinjection buffer (20 mM Hepes, pH 7.4/20 mM NaCl/4 mM MgCl2/0.5 mM 2-mercaptoethanol). His-Ras preparations (at least 70% pure) were concentrated using Centriprep-10 concentrators (Amicon) and stored at −70°C in microinjec-

Abbreviations: MAP kinase, mitogen-activated protein kinase; p42MAPK, 42-kDa MAP kinase; MPF, maturation-promoting factor; GVBD, germinal vesicle breakdown; MBP, myelin basic protein; CHX, cycloheximide.
tion buffer plus 40% glycerol. The purification of His6-ERK2 from *E. coli* carrying the NTP7-5 vector was performed using identical conditions, but without dodecyl β-maltoside. Recombinant clam cyclins AΔ60 and BΔ97 were expressed and gel-purified as described (33).

**Frog Oocyte Maturation Assays.** Oocyte isolation, micro-injection, and culture were as described (33). Oocytes were microinjected with 50 nl and germline vesicle breakdown (GVBD) was scored by the appearance of a white spot at the animal pole. For immunoblot analysis, 4 vol of lysis buffer (0.25 M sucrose/0.1 M NaCl/2.5 mM MgCl2/10 mM NaF/10 mM EGTA/1.0 mM Na3VO4/20 mM Hepes, pH 7.2) was added to 1 vol of packed oocytes (0.9 μl per oocyte), and oocytes were homogenized by repeated passage through a plastic pipette tip. The homogenate was centrifuged at 10,000 × g for 5 min and mixed with 4 vol of 2X sample buffer (34).

**Preparation of Oocyte and Egg Extracts.** Defolliculated, late stage V and VI oocytes, ≥1.2 mm (35), were isolated from ovaries by collagenase treatment (36, 37), rinsed twice in extraction buffer (EB: 0.25 M sucrose/0.1 M NaCl/2.5 mM MgCl2/20 mM Hepes, pH 7.2), and then transferred to a 5-ml centrifuge tube containing EB with pepstatin, chymostatin, and leupeptin (each at 10 μg/ml). Excess EB was removed and oocytes were crushed by centrifugation at 15,000 × g for 15 min in a swinging bucket rotor at 2°C. The supernatant, between the lipid cap and packed yolk was collected and cytochalasin B was added (50 μg/ml) before a second centrifugation under the same conditions. Aliquots were frozen in liquid nitrogen and stored at −80°C. Fresh and frozen/thawed extracts responded equally well to added proteins. Ovulated, metaphase II-arrested eggs were obtained and dejeled (38). Extracts were prepared from unactivated eggs as described for oocytes except that the EB contained 10 mM NaF (recrystallized, Sigma) and 10 mM EGTA (Sigma). To prepare cycloheximide (CHX)-arrested extracts, dejelled and activated metaphase II-arrested eggs were incubated in 2% modified Steinberg’s solution (39) with CHX (100 μg/ml, Sigma) for 15 min, electrically activated (40), and then cultured in this solution for 45 min at 21°C. Extracts were prepared as described for oocytes.

**In Vitro Assays of p42MAPK Activation.** Assay samples (25–60 μl) were prepared by mixing 16 vol of extract, 1 vol of an ATP regeneration system [20 mM ATP, 20 mM MgCl2, 0.2 M creatine phosphate, and creatine kinase (1 mg/ml, Boehringer Mannheim) in EB], and 3 vol of EB or other additions. For assay of Ras, 5.0-mg/ml preparations were diluted with 1 vol of dilution buffer [50 mM NaCl/2 mM MgCl2/20 mM Hepes, pH 7.4 (20)] and added to extracts at a dilution of 1:19, to yield a final concentration of 125 μM/μl. Purified His6-Ras proteins were subjected to a GTP-exchange reaction by mixing 1 vol of protein from preparation (5.0 mg/ml) with 1 vol of exchange buffer (50 mM NaCl/1 mM dithiothreitol/10 mM EDTA/2 mM GTP/50 mM Tris-HCl, pH 7.5) and incubating at 37°C for 10 min, before addition to extracts. In addition, all Ras assays included 1 mM GTP. For assay of cyclins, samples included 3 vol of 40 mM Tris acetate, pH 7.2 with either cyclin AΔ60 (0.15 mg/ml) or cyclin BΔ97 (0.25 mg/ml), to yield final concentrations in extracts of 0.5 μM and 0.9 μM, respectively. Okadaic acid was added to a final concentration of 1.0 μM in samples. For *in vitro* activation of His6-ERK2, the protein was added to extracts to yield a final concentration of 150 μg/ml. After mixing on ice, samples were incubated at 21°C and aliquots were analyzed by immunoblotting (1 vol of extract/10 vol of sample buffer) or kinase assays (1 vol of extract/10 vol of lysis buffer). Metaphase II-arrested egg extract was diluted with EB and sample mix was prepared in the same manner.

**Immunoblotting.** Immunoblotting with MAP kinase antibodies (anti-ERK1 691 and 837; ref. 41), phosphotyrosine antibodies (42), and affinity-purified cdc2 antibodies was as described (33, 43), using alkaline phosphatase-conjugated secondary antibody (Promega).

**Isolation of His6-ERK2 and Endogenous cdc2 from Extracts, and Kinase Assays.** To recover His6-ERK2 from the extracts, 10 μl of extract was mixed with 20 μl of EB/10 mM NaF/1 mM Na3VO4/120 mM imidazole and then frozen in liquid nitrogen. Pro-Bond resin was prepared as a 1:1 slurry in EB/10 mM NaF/1 mM Na3VO4 and 20 μl was added to each thawed sample and mixed for 30 min at 4°C. The pelleted resin was then washed once in EB containing 0.5 M NaCl, 40 mM imidazole, 0.2% Tween 20, 0.2 mM Na3VO4, and 10 mM NaF; twice in the same buffer, but with 0.1 M NaCl; and finally, once in this buffer without Tween 20. The resin was suspended in 40 μl of final wash buffer, and 5 μl was assayed for kinase activity with myelin basic protein (MBP) as substrate. The remaining resin was pelleted and bound protein was eluted with 30 μl of sample buffer for analysis by immunoblotting. cdc2 kinase was isolated on p13 nucleotide. Sepharose (33), using the same buffers as those used for His6-ERK2 isolation, but without imidazole. Histone H1 kinase or MBP kinase assays of samples were done as described (43). H1 bands were excised from dried gels and immersed in Aquisol (DuPont), and 32P incorporation was quantitated in a scintillation counter. The in-the-gel kinase assay was as described (16, 44).

**RESULTS**

**Ras-Induced Oocyte Maturation Is Accompanied by Tyrosine Phosphorylation of p42MAPK.** Injection of human oncoprotein [Val12]Ha-Ras into quiescent frog oocytes leads to resumption of the meiotic cell cycle as judged by GVBD. With the relatively low-specific-activity preparation used for Fig. 1, only the highest amount of Ras (100 ng) induced GVBD in a significant number of oocytes (40%; data not shown). To monitor the status of p42MAPK, lysates were made from oocytes before or 5 hr after injection or progesterone treatment and analyzed by immunoblotting. Quiescent oocytes contained a 42-KDa protein that was recognized by MAP kinase antibodies but not by phosphotyrosine antibodies (Fig. 1). Both progesterone-matured and Ras-matured oocytes showed almost complete conversion of the p42MAPK to a more slowly migrating form that was recognized by phosphotyrosine antibodies and represented, as previously shown (14, 45, 46), the active form of the enzyme. Both also showed the tyrosine dephosphorylation of a 34-KDa protein, identified here (see below) and elsewhere (47–49), as cdc2 kinase. In this system and others, tyrosine dephosphorylation of cdc2 is a reliable marker of its activation (50–53). A considerable fraction of p42MAPK was tyrosine-phosphorylated even in the Ras-injected oocytes, which did not undergo GVBD. However, there was no significant tyrosine dephosphorylation of cdc2 in these oocytes, suggesting that p42MAPK activation neither depends on nor follows cdc2 activation.

**Ras- and Cyclin-Induced Activation of p42MAPK in Extracts of Quiescent Oocytes.** To test if cell cycle activators could induce p42MAPK activation in an extract of quiescent oocytes, a 15,000 × g extract was prepared and supplemented with GTP and an ATP-regenerating system. Both p42MAPK (Fig. 2 A and B) and cdc2 (Fig. 2 C) remained inactive for up to 4 hr of incubation at 21°C. The addition of [Val12]Ras induced the activation of p42MAPK 2–3 hr later, as judged by the shift in its electrophoretic mobility, coincident with the appearance of both a phosphotyrosine signal at 42 KDa (Fig. 2 A) and a 42-KDa-associated kinase activity toward MBP (Fig. 2 B). Boiled Ras had no activity (data not shown). Although His6-[Val12]Ras had activity comparable to untagged [Val12]Ras, nononcogenic His-[Gly]12[Ras was a much less effective activator of p42MAPK in oocyte extracts (Fig. 2 D). His6-[Val12,Arg16]Ras, a mutant that cannot undergo pre-
While [Val^{22}]Ras led to activation of p42MAPK, it did not induce the tyrosine dephosphorylation nor the activation of cdc2 (Fig. 2C). In contrast, 1 μM okadaic acid induced rapid activation of both p42MAPK and cdc2 in oocyte extracts (Fig. 2A–C), just as in intact oocytes (56).

Both clam cyclins, Aα60 and BΔ97, also led to activation of p42MAPK in oocyte extracts (Fig. 3). As judged by the appearance of a phosphotyrosine signal at 42 kDa, p42MAPK activation was first detected 60 min after cyclin addition. In contrast to the Ras-induced activation of p42MAPK, cyclin-induced p42MAPK activation was preceded by activation of cdc2 kinase (as measured by histone H1 kinase activity) and dephosphorylation of the cdc2 subunit of pre-MPF (Fig. 3).

A recombinant MAP kinase tagged with oligohistidine (His_{6}-ERK2) was also an effective target for Ras-induced activation. Like other recombinant MAP kinases (57–59), His_{6}-ERK2 showed a low level of endogenous phosphorylation. When added to the quiescent oocyte extract, it remained inactive for at least 4 hr, during which time it was dephosphorylated (Fig. 4). The addition of [Val^{22}]Ras to the extracts led to moderate tyrosine phosphorylation and activation of His_{6}-ERK2. In oocyte extracts stimulated by okadaic acid, His_{6}-ERK2 was completely converted to the upper, activated form. Most strikingly, extracts of metaphase II-arrested oocytes, which contain high levels of active, endogenous p42MAPK and MAP kinase kinase (15, 16, 46, 60, 61), showed a very rapid activation of the added His_{6}-ERK2, with >50% of the protein being shifted to the upper, active form even at 4°C (Fig. 4).

Ras- and Cyclin-Induced Activation of p42MAPK in Extracts of CHX-Arrested Interphase Cells. Purified MPF can lead to activation of frog p42MAPK, both during oocyte maturation in vivo and in extracts of fertilized eggs. In contrast, injection of active p42MAPK into quiescent oocytes does not lead to cdc2 activation or meiotic maturation (14, 60, 61). From these results, it has been suggested that p42MAPK activation both follows and is dependent on cdc2 activation. To test this idea,
we made extracts that contained inactive cdc2 (40, 55, 62). To prepare these, metaphase II-arrested eggs were treated with CHX and then electrically activated to trigger entry into interphase of the first mitotic cell cycle. At 45 min postactivation, when endogenous cyclins had been destroyed, cdc2 activity had disappeared, and new cyclin synthesis had been blocked, eggs were collected and an extract was made. The addition of His6-[Val12]Ras to CHX extracts led to the activation of p42MAPK in the complete absence of cdc2 activation (Fig. 5). Okadaic acid also stimulated p42MAPK, but with less than a 2-fold increase in H1 kinase activity (not shown). In contrast, addition of either cyclin AΔ60 (Fig. 5) or cyclin BΔ97 (data not shown) activated both cdc2 and p42MAPK, with cdc2 activation preceding that of p42MAPK. Thus, p42MAPK can be activated in extracts of oocytes and mitotic interphase cells by two pathways, a Ras-dependent pathway that does not require activation of cdc2 and a cyclin-activated cdc2-dependent pathway.

**DISCUSSION**

We have developed a cell-free system from Xenopus oocytes which reproduces the Ras-induced activation of p42MAPK that occurs when intact oocytes and other quiescent cells are stimulated to reenter the cell cycle. The lag between Ras addition and p42MAPK activation may reflect the need for the processing of Ras, which is slow even in vivo (20). Ras proteins normally undergo a complex set of modifications, resulting in the cleavage, isoprenylation, methylation, and palmitoylation of the C terminus of the protein concomitant with its membrane localization (63). Whether or not this processing is responsible for the delay in p42MAPK activation in vitro, processing is most likely required, since [Val12, Arg186]Ras, a mutant that lacks the C-terminal Cys-Ala-Ala-Xaa (Ala, aliphatic residue) motif (64), neither undergoes processing nor induces p42MAPK activation. Although p42MAPK is activated by Ras both in vivo and in vitro, we have not observed any Ras-induced cdc2 activation in vitro. These results show that the activation of oocyte p42MAPK by Ras does not occur via cdc2 activation. Either oocyte extracts lack the capacity for Ras-induced cdc2 activation or, alternatively, activation of cdc2 by Ras in vitro may be much slower than that of p42MAPK and, therefore, not observed during the limited time monitored in these studies.

The oocyte cell-free system also reproduces two other events that can lead to cell cycle reentry in vivo, activation of cdc2 by cyclins (22) and the phosphatase inhibitor okadaic acid (56). Oocytes contain cdc2, some of which is complexed with cyclin B and kept inactive by phosphorylation of cdc2 on tyrosine (reviewed in ref. 18). Hormonal stimulation induces dephosphorylation and subsequent activation of cdc2 by a homolog of the fission yeast phosphatase cdc25. Although cdc2 dephosphorylation is thought to proceed via an autocatalytic amplification loop involving cdc25 (reviewed in refs. 65 and 66), it is not clear what triggers this loop during normal, hormone-induced maturation. Our observation that cyclins induce the tyrosine dephosphorylation of endogenous cdc2 in oocyte extracts suggests that added cyclins bind free cdc2, forming active complexes that trigger activation of cdc25, which, in turn, dephosphorylates and activates the endogenous cdc2/cyclin B complexes.

**Fig. 3.** Clam cyclins AΔ60 and BΔ97 induce activation of both cdc2 and p42MAPK in oocyte extracts. Extracts were incubated with cyclins and samples were analyzed by blotting as in Fig. 2A with antibodies against phosphotyrosine (α-p-tyr) (Top) or MAP kinase (α-ERK) (Middle). Samples were also assayed for histone H1 kinase activity (Bottom).

**Fig. 4.** In vitro activation of exogenous, recombinant MAP kinase. Oocyte or metaphase II-arrested egg (MII) extracts were incubated for 0 or 4 hr as follows: oocyte extract plus His6-ERK2; oocyte extract alone; oocyte extract plus 1 μM okadaic acid (OA) and His6-ERK2; MII extract plus His6-ERK2. His6-ERK2 was recovered from the lysates by binding to Pro-Bond resin and blotted with antibodies against phosphotyrosine (α-p-tyr) (Top) or MAP kinase (α-ERK) (Middle) and assayed for MBP kinase activity (Bottom).

**Fig. 5.** Both cyclin and [Val12]Ras induce activation of p42MAPK in extracts of CHX-arrested interphase cells. Extracts of CHX-arrested activated eggs were incubated with buffer, cyclin ΔA, or His6-[Val12]Ras, and aliquots were removed at the indicated times (hr). Samples were analyzed as in Fig. 3. (Top) Anti-phosphotyrosine blot. (Middle) Anti-ERK blot. (Bottom) Histone H1 kinase activity.
The normal pathway for p42MAPK activation in vivo is not clear. A pathway for p42MAPK activation can be triggered, at least experimentally, by introduction of MPF into intact oocytes and fertilized egg extracts (14, 60, 61) or, as shown here, by the addition of purified cyclins to oocyte and embryonic extracts, yet the Ras-induced activation of p42MAPK in these extracts occurs without any detectable activation of cdc2. Similarly, in G3-arrested somatic cells, Ras-induced activation of p42MAPK proceeds without any apparent involvement of cdc2, since such cells lack significant cdc2 protein or activity (67).

The establishment of a cell-free assay from vertebrate cells for Ras-induced p42MAPK activation should facilitate the dissection of this pathway. Oocyte and egg extracts can be prepared in large quantities, which may allow the biochemical isolation of components of this pathway and the discovery of their functions. In addition, specific hypotheses about the roles of other proteins involved in the modulation of Ras can now be tested directly.

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