Reconstitution of p21\textsuperscript{ras}-dependent and -independent Mitogen-activated Protein Kinase Activation in a Cell-free System*

(Received for publication, July 7, 1993)

Barbara VanRenterghem, Jackson B. Gibbs, and James L. Maller

From the \$Howard Hughes Medical Institute and Department of Pharmacology, University of Colorado School of Medicine, Denver, Colorado 80262 and the \$Department of Cancer Research, Merck Research Laboratories, West Point, Pennsylvania 19486

In Xenopus oocytes, mitogen-activated protein (MAP) kinase can be activated by progesterone treatment or by microinjection of cyclin A, both of which lead to activation of the cdc2 protein kinase. The tyrosine kinase pp60\textsuperscript{src} has previously been shown to accelerate progesterone-induced oocyte maturation and to increase the phosphorylation of ribosomal protein S6 by pp90\textsuperscript{raf}, most likely by activating cyclin-dependent protein kinase activity of MPF (36, 37). These results have shown the steps downstream of G-protein-coupled signals (20, 21). Recently a cell-free oocyte extract has been shown to be responsive to p21\textsuperscript{H-ras} for activation of MEK and MAPK but not for activation of the H1 kinase activity of MPF (36, 37). These results have shown the steps downstream of p21\textsuperscript{H-ras} can be reconstituted in this system. We show here that in oocyte extracts tyrosine kinase activation of MEK and MAPK also can be reconstituted. Furthermore, cdc2 and pp60\textsuperscript{src} initiate two different signal transduction mechanisms that converge to activate MEK but are divergent at a step upstream of p21\textsuperscript{H-ras} activation.

Mitogen-activated or extracellular signal-regulated protein kinases (MAPKs)\textsuperscript{1} comprise a family of serine/threonine-specific protein kinases activated in various cell types (1–3) in response to tyrosine kinase-coupled signals (4–7), G-protein-coupled signals (8–10), protein kinase C (11, 12), and progesterone in Xenopus oocytes (13–16). The effect of MAPK activation is evident in direct activation of the Rsk family of S6 kinases and in phosphorylation of transcription factors involved in mitogenic stimulation (5, 17–19). Recently, much emphasis has been placed on the upstream regulators of MAPK, and a cascade involving several MAPK kinases (MEKs) and MAPK kinase kinases (MEKKs) and MAPK kinase kinases (MEKKs) has been reported (20–27).

It is known that p21\textsuperscript{ras} is required for activation of MAPK by nerve growth factor and protein kinase C (12, 28) and that either anti-Ras antibodies or a dominant-negative mutant of c-Ha-ras (\textasciitilde{Asn}\textsuperscript{17}Ha-ras) can block insulin-, nerve growth factor-,

\textsuperscript{1} This work was supported by National Institutes of Health Grants GM26743 and DK28853. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

\textsuperscript{1} Investigator of the Howard Hughes Medical Institute. To whom correspondence should be addressed.

The abbreviations used are: MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated protein kinase; EGF, epidermal growth factor; MEK, MAPK kinase; MPF, maturation-promoting factor; EGFR, EGF receptor; GAP, GTPase-activating protein; IGF-1, insulin-like growth factor 1.


tor-, and v-Src-induced signaling in a variety of systems (25–35). Recently, p21\textsuperscript{ras} function has also been shown to be critical for MAPK regulation in Xenopus oocytes (36, 37). In addition, there is genetic evidence in both C. elegans and D. melanogaster for p21\textsuperscript{ras} activation downstream of a tyrosine kinase signal (38–40).

While various cell types may emphasize particular signaling mechanisms, in order to understand integrated cell behavior, it is important to determine which MEKs and MEKKs are involved in various signal transduction pathways in a single cell and to elucidate where different pathways may converge in the ultimate activation of MAPK and pp90\textsuperscript{raf}. Johnson and coworkers (20) showed that different MEKs act downstream of different signals initiated at the cell surface. The Raf proto-oncogene kinase in the tyrosine kinase pathway is thought to be a direct MEKK (22, 23, 41), but a distinct MEKK is downstream of G-protein-coupled signals (20). It has been suggested recently that the c-mos proto-oncogene kinase can also function as a MEKK in vitro (42). Xenopus oocytes are a good model system in which to study MAPK regulation by multiple signals in a single cell. MAPK has been shown to be activated in this system by progesterone treatment (16, 43), by microinjection of p21\textsuperscript{H-ras} (36, 37), cyclin (13, 33, 37), or maturation-promoting factor (MPF), a complex of cdc2 and cyclin B (25, 44, 45). The tyrosine kinase pp60\textsuperscript{src} has been found to increase the phosphorylation of ribosomal protein S6 (46) (a consequence of MAPK activation of pp90\textsuperscript{raf} (5, 16)). Recently a cell-free oocyte extract has been shown to be responsive to p21\textsuperscript{H-ras} for activation of MEK and MAPK but not for activation of the H1 kinase activity of MPF (36, 37). These results have shown the steps downstream of p21\textsuperscript{H-ras} can be reconstituted in this system. We show here that in oocyte extracts tyrosine kinase activation of MEK and MAPK also can be reconstituted. Furthermore, cdc2 and pp60\textsuperscript{src} initiate two different signal transduction mechanisms that converge to activate MEK but are divergent at a step upstream of p21\textsuperscript{ras} activation.

**EXPERIMENTAL PROCEDURES**

**Materials—** The v-src baculovirus construct was a gift from Dr. Helen Powne-Worms (Harvard Medical School), and purification of pp60\textsuperscript{src} was carried out by immunoaffinity chromatography as previously described (47). The bovine protein A-cycA fusion construct was a gift from Dr. Tim Hunt, and the product was isolated from bacteria overexpressing the protein (48). MFH was purified by p13-Sepharose chromatography as described in Ref. 49. MEK antibody was made in rabbits against a synthetic peptide encoding the first 15 residues of the N terminus of the MEK protein (50, 51). Inhibitor of cAMP-dependent protein kinase was prepared by E. Erikson in this laboratory by the method of Ref. 52. The wild-type and kinase-negative (K63M) GST-Erk constructs (21) were a gift from Dr. R. L. Erikson (Harvard University) and the protein products were purified by glutathione-agarose affinity chromatography. The construction and preparation of p21\textsuperscript{ras} proteins has been described previously (53). RAS\textsuperscript{1}, formerly called [Leu\textsuperscript{66}RAS1term.185] has a dominant-negative phenotype. It lacks the CAAX box (where X is any amino acid) and X is any amino acid, and the Leu\textsuperscript{66} mutation results in binding of GTPase-activating protein (GAP) with a 100-fold higher affinity than [Val\textsuperscript{66}Ras in vitro (53). The EGF receptor peptide (EGFR(662-681)) for use as a MAPK substrate (54) was synthesized by a local peptide synthesis facility.

**Oocyte Dissection and Preparation of Extracts—** X. laevis stage VI oocytes were isolated by modification of previously described methods (27, 28) in 6 volumes of 80 mM \(\beta\)-glycerophosphate, 20 mM EGTA, 15 mM MgCl\(_2\), 20 mM Tris, pH 7.2, 1 mM phenylmethylsulfonyl fluoride, and 3 \(\mu\)g/m\(\ell\) leupeptin. Homogenates were spun at 12,000 \(\times\) g for 5 min and the supernatant fraction removed and diluted 10-fold with 20 mM Tris, pH

19935
Activation of MAP Kinase in a Cell-free System

RESULTS

Extracts from resting oocytes were prepared as described under "Experimental Procedures" and examined for the activation of MAPK following addition of various amounts of cyclin A, pp60surf, or MPF (Fig. 1). All three agents were able to cause activation of MAPK, and cyclin A also caused activation of H1 kinase activity. Activation of H1 kinase activity by cyclin A was not blocked by vanadate, and endogenous cyclin B2 did not become phosphorylated (data not shown), suggesting cyclin A directly associates with cdc2 and expresses H1 kinase activity without activating the endogenous oocyte store of tyrosine phosphorylated pre-MPF. The EC50 value of cyclin A for MAPK and H1 K activations is approximately 500 nM (Fig. 1, panels A and B). The EC50 value of pp60surf for stimulating MAPK activity is approximately 3 units (Fig. 1, panel C). The effects of pp60surf were also evident in the presence of cycloheximide, confirming an activation mechanism. However, pp60surf did not stimulate H1 K activity in these extracts (data not shown). One unit of pp60surf or MPF catalyzes incorporation of 1 pmol of phosphate/min into casein or calf thymus histone H1, respectively.

Because tyrosine kinase-mediated activation of MAPK in intact somatic cells is thought to require a functional Ras protein (28–35), the effect of a dominant-negative Ras protein on these activations was studied. The dominant-negative Ras protein used, termed RAST, lacks a CAAX box required for acylation and membrane localization and has a leucine at position 68, leading to a 100-fold increase in GAP binding (53).

The effect of RAST on the stimulatory effects of cyclin A, pp60surf, and [Val12]pp21Ha-ras is shown in Fig. 2. Cyclin A was able to stimulate MAPK activity 3–4-fold over control levels in a time-dependent manner regardless of whether extracts had been preincubated with dominant-negative RAST (Fig. 2, panel A). Similarly, pp60surf was able to stimulate MAPK activity 3–4-fold over control levels (Fig. 2, panel B), but preincubation with RAST inhibited the stimulation of MAPK activity (Fig. 2, panel B). Ras protein could also stimulate MAPK activity 2–3-fold over control levels (Fig. 3, panel C; Refs. 36 and 37), in a manner inhibited by preincubation of extracts with RAST but not by preincubation with an effector mutant of RAST that cannot bind GAP as a result of an additional mutation in the effector domain (Ala45-RAS; Fig. 2, panel C).

Fig. 3 shows that inhibition of pp60surf stimulation of MAPK activity required RAST to be present for at least 10 min before pp60surf addition. When RAST and pp60surf were added together at time zero, only slight inhibition of MAPK activity was evident. At least 10 pg/ml RAST was required to inhibit the stimulation of MAPK by pp60surf, with an approximate IC50 value of 7.5 µg/ml (Fig. 3).

Since both cyclin A and pp60surf were capable of stimulating MAPK, it was important to determine whether these stimulators could also activate MEK. Xenopus MEK has been purified, cloned and sequenced by Nishida and co-workers (25, 51). Using an antibody against the first 15 residues of the protein, MEK was immunoprecipitated from treated oocyte extracts and assayed for phosphorylation of a mammalian kinase-inactive MAPK fusion protein (GST-Erk) as substrate. Fig. 4 (panels A and B) shows that both cyclin A and pp60surf stimulated MEK in these extracts. Since cyclin A is thought to bind cdc2 and form an active H1 kinase after microinjection into oocytes or addition to egg extracts (33, 37, 56–58), it seemed likely that similar results would be obtained upon addition of purified MPF (cdc2/cyclin A) to the extract. MPF and cyclin A stimulated MEK activity in these extracts to similar levels, suggesting that the cyclin A is acting in these extracts by forming a complex with cdc2. This result is consistent with evidence that cyclin A does not associate significantly with cdc2 in Xenopus oocytes or eggs (59). It also indicates both cyclin A and cyclin B...
Activation of MAP Kinase in a Cell-free System

The results in this paper show that cyclin A/cdc2, pp60<sup>src</sup>, and MAPK (cyclin B/cdc2) can activate both MEK and MAPK in Xenopus oocyte extracts. These pathways appear to have distinct intermediates that converge on MEK because dominant-negative RAS<sup>T</sup> can inhibit the ability of pp60<sup>src</sup> to activate MAPK but has no effect on activation by cyclin A. Although the ability of p21<sup>ras</sup> to cause MAPK activation in oocyte and egg extracts has been observed before (25, 37), the present results are the first demonstration that events between tyrosine kinase activation and Ras that are required for MAPK activation can also be reconstituted in a cell-free system. The ability of cyclin A/cdc2 and cyclin B/cdc2 to cause activation appears to be a direct effect in these experiments rather than an indirect one resulting from activation of oocyte pre-MPF. The activation of pre-MPF by elevated cyclin-dependent kinase activity in other experiments (37) may be a consequence of different protocols for extract preparation.

Our results show that oocyte extracts are capable of completing a tyrosine kinase-initiated pathway for MAPK in terms of events both upstream and downstream of p21<sup>ras</sup>. The data presented here in extracts are consistent with previous results that show oocyte maturation, which is invariably accompanied by MAP kinase activation (16), is blocked by anti-p21 antibodies when induced by insulin or IGF-1 (29, 34). However, maturation induced by progesterone is unaffected by anti-p21 reagents. The present results raise the possibility that at least part of the mechanism by which anti-Ras reagents block tyrosine kinase-initiated oocyte maturation involves failure to activate the MAP kinase pathway.

Previous studies have compared the activation of S6 kinase II by tyrosine kinase-initiated signals versus p21<sup>ras</sup> or progesterone (15, 60, 61). Those studies are very likely to have also reflected activation of MAPK, since fully activated S6 kinase can be prepared in vitro with MAP kinase, and the kinetics of S6 kinase activation closely parallel MAPK activation (16). Insulin/IGF-1 treatment of oocytes leads to a rapid transient activation of S6K, followed several hours later by activation of MAPK and a larger increase in both MAP kinase and S6 kinase activity (61). The early increase in S6 kinase activation, but not the later one, is resistant to protein synthesis inhibition. It seems likely that the increase in MAP kinase and S6 kinase activation occurring at the time of MAPK activation reflects an MPF/cdc2-mediated, p21<sup>ras</sup>-independent activation, whereas the earlier transient increase with insulin/IGF-1 reflects a tyrosine kinase-initiated, p21<sup>ras</sup>-dependent, cyclin/cdc2-independent pathway. Both of these pathways can now be reconstituted in extracts from resting oocytes and directly compared in vitro.

Little is known about how cdc2 kinase can initiate a pathway leading to activation of MEK and MAPK. However, rapid progress is being made on identification of intermediates between a tyrosine kinase and p21<sup>ras</sup>. Intermediates identified so far include "adapter" molecules such as GRB2 with SH2 domains that bind to specific phosphotyrosyl residues on activated tyrosine kinases and guanine nucleotide exchange factors such as Son of Sevenless (Sos) (63-65). Thus, intermediate proteins such as GRB2 and Sos are likely to be active in the oocyte extract system.

In this study neither Ras nor Src appear to be upstream of a cyclin/cdc2 signal as neither can stimulate H1 kinase activity in these extracts (data not shown). Therefore, the evidence suggests that pp60<sup>src</sup> and cdc2 initiate two different signal transduction pathways: one involving a p21<sup>ras</sup>-independent signal from cyclin/cdc2 through MEK to MAPK and the other involving pp60<sup>src</sup> through Ras to MEK, perhaps via c-Raf (22, 23, 41) or c-Mos (42), and then to MAPK. The involvement of Ras in the activation of MEK and MAPK by pp60<sup>src</sup> supports previous findings in intact cells and extends these results by...
reconstituting, in vitro, the steps between tyrosine kinase activation and Ras in this mitogenic signaling system. The results also show that two different signaling pathways that diverge upstream of Ras activation can be directly compared in vitro. This development of a cell-free system for tyrosine kinase signaling that faithfully performs steps both upstream and downstream of p21ras should enhance the ability to biochemically dissect and reconstitute this mitogenic pathway.

Acknowledgments—We thank Andrea Lewellyn and Jan Kyes for technical assistance and Eleanor Erickson for valuable discussions.

REFERENCES


FIG. 4. MEK stimulation by cyclin A, ppp60′′′(E), and MPF. A, oocyte extract, treated with either control protein A (PrA), 650 μg protein A-cyclin A (ccycl A), or buffer alone (-E) for 30 min, was assayed for MEK activity by immunocomplex assays with R63M GST-Erk as substrate. B, MEK was immunoprecipitated from extracts treated either with control buffer (B), 7.5 units of ppp60′′′(E)-src), or buffer alone (-E) and asayed as described in A. C, MEK was immunoprecipitated from extracts treated either with control buffer (B), 1 unit of MPF (MPF), or buffer alone (-E) and assayed as described in A. Results are representative of three independent experiments.