A Constitutively Active Form of the Protein Kinase p90<sup>Rsk1</sup> Is Sufficient to Trigger the G<sub>2</sub>/M Transition in Xenopus Oocytes*

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The protein kinase p90<sup>Rsk1</sup> has previously been implicated as a key target of the MAPK pathway during M phase of meiosis II in Xenopus oocytes. To determine whether Rsk is a mediator of MAPK for stimulation of the G<sub>2</sub>/M transition early in meiosis I, we sought to generate a form of Rsk that would be constitutively active in resting, G<sub>2</sub> phase oocytes. Initial studies revealed that an N-terminal truncation of 43 amino acids conferred enhanced specific activity on the enzyme in G<sub>2</sub> phase, and stability was highest if the C terminus was not truncated. The full-length enzyme is known to be activated by phosphorylation at five sites. Two of these sites and flanking residues were replaced with either aspartic or glutamic acid, and Tyr<sup>985</sup> was mutated to alanine. The resulting construct, termed fully activated (FA) Rsk, had constitutive activity in G<sub>2</sub> phase, with a specific activity equivalent to that of wild type Rsk in M phase. In eight independent experiments ~45% of oocytes expressing FA-Rsk underwent germinal vesicle breakdown (GVBD, the G<sub>2</sub>/M transition) in the absence of progesterone, and this effect could be observed even in the presence of the MAPK kinase inhibitor U0126. Moreover, the specific activity of FA-Rsk in vivo was unaffected by U0126. In oocytes that did not undergo GVBD with FA-Rsk expression, subsequent treatment with progesterone resulted in a very rapid rate of GVBD even in the presence of U0126 to inhibit the endogenous MAPK/Rsk pathway. These results indicate that Rsk is the mediator of MAPK effects for the G<sub>2</sub>/M transition in meiosis I and in a subpopulation of oocytes Rsk is sufficient to trigger the G<sub>2</sub>/M transition.

Xenopus oocytes undergo the G<sub>2</sub>/M transition (oocyte maturation) in vitro in response to progesterone. During maturation several signal transduction pathways are activated, including the polo-like kinase pathway and the MAPK<sup>1</sup> pathway (see Refs. 1 and 2 for review). The role of the MAPK pathway in oocyte maturation has been studied at several points in the process. At the end of maturation the cell cycle is arrested in metaphase of meiosis II by a MAPK-dependent activity known as cytostatic factor (CSF) (3). The substrate of MAPK that mediates CSF arrest appears to be the protein kinase p90<sup>Rsk1</sup> (Rsk), inasmuch as activated Rsk causes CSF arrest even when MAPK is inactive (4), and depletion of Rsk from extracts removes CSF activity (5). Rsk may mediate this function by activating the protein kinase Bub1 (6), a component of the spindle assembly checkpoint pathway in the cell cycle, leading to inhibition of the anaphase-promoting complex (APC), an E3 ubiquitin ligase that targets cyclin B for degradation in anaphase (see Ref. 7 for review). Another MAPK-dependent transition in the cell cycle, the linkage of M phases at the meiosis I → II transition, is also accounted for by activation of Rsk and subsequent inhibition of APC-mediated cyclin B degradation (8).

The third cell cycle transition in maturation regulated by the MAPK pathway is the G<sub>2</sub>/M transition upon entry into meiosis I in response to progesterone. MAPK is activated after progesterone treatment as a consequence of new synthesis of the c-Mos proto-oncogene product, a MAPK kinase kinase (for review, see Ref. 9). MAPK plays an important role in entry into meiosis I as judged by both gain-of-function and loss-of-function experiments. Inhibition of Mos translation or inhibition of MAPK kinase leads to delays in the rate of maturation and/or a reduction in the fraction of oocytes able to undergo GVBD (10–12). On the other hand, overexpression of Mos, activated MAPK kinase 1, or activated MAPK can cause GVBD in the absence of progesterone in a large fraction of the oocyte population (13–16). Inasmuch as the effects of the MAPK pathway in M phase of meiosis II are mediated by Rsk (4, 5, 8), the present study was undertaken to investigate whether Rsk also mediates the effects of the MAPK pathway in G<sub>2</sub> phase governing entry into meiosis I.

MATERIALS AND METHODS

Mutations were introduced into the FLAG-tagged Xenopus Rsk1 sequence (17) by polymerase chain reaction using the QuikChange site-directed mutagenesis kit (Stratagene). All mutations were confirmed by DNA sequencing. mRNA encoding various constructs was transcribed using a mMessage Machine kit (Ambion), and 50 nl of 1 mg/ml mRNA was injected into each oocyte as described previously (4). Immune complex kinase assays with anti-FLAG beads (Sigma) were performed as described previously using S6 peptide as substrate (4, 18). To determine the specific activity of various Rsk constructs, anti-FLAG immune precipitates were immunoblotted with anti-FLAG antibodies, and kinase activity was normalized for level of expression. All samples from a particular experiment were blotted on the same gel, and control experiments demonstrated that the FLAG antibody beads completely depleted all recombinant protein from the extracts. GVBD was monitored initially by formation of a well defined white spot in the animal pole of the oocyte and was generally confirmed biochemically using procedures for assaying histone H1 kinase activity, cyclin B2 electro...

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The abbreviations used are: MAPK, mitogen-activated protein kinase; CSF, cytostatic factor; APC, anaphase-promoting complex; GVBD, germinal vesicle breakdown; PDK-1, 3-phosphoinositide-dependent kinase-1; FA, fully activated; WT, wild type; GSK-3, glycogen-synthase kinase 3.
phoretic mobility shifts, and Cdc2 Tyr215 phosphorylation that have been described previously (4, 12, 18).

RESULTS

Our general approach to investigate Rsk function in the induction of maturation was to generate a form of Rsk that would be constitutively active in the G2 environment of a resting oocyte. These efforts took advantage of the evidence that activation of the Rsk N-terminal kinase domain requires phosphorylation of specific sites by 3-phosphoinositide-dependent kinase-1 (PDK-1), MAPK, and the Rsk C-terminal kinase domain (see Ref. 19 for review). Previous efforts investigating Rsk structure/function relationships had led to generation of a Rsk construct lacking the C-terminal kinase domain, termed CA-Rsk, that had significant constitutive activity in M phase of meiosis II even in the presence of a potent inhibitor of the MAPK pathway, U0126 (4, 8). Although CA-Rsk is active in M phase without an active MAPK pathway (4, 8), it is much less constitutive activity, two MAPK phosphorylation sites in the N-terminal kinase domain, Thr570 and Ser576, that appear to contribute to activation (24). As an approach for generating constitutive activity, two PDK-1 phosphorylation sites in the linker region, Thr568 and Ser572, that appear to contribute to activation (24). As an approach for generating constitutive activity, two PDK-1 phosphorylation sites in the linker region were mutated to aspartic acid (23). Fig. 2 shows the phosphorylation sites involved in Rsk activation (24). The N-terminal kinase domain requires phosphorylation of the activation loop at Ser220 by PDK-1 (4, 25, 26). This event is facilitated by the binding of PDK-1 to Ser378 in the linker region after it has been phosphorylated by the C-terminal kinase domain (27). Activation of the C-terminal kinase domain requires phosphorylation by MAPK of Thr570 in the T-loop (24). MAPK also phosphorylates two residues in the linker region, Thr568 and Ser572, that appear to contribute to activation (24). As an approach for generating constitutive activity, two PDK-1 phosphorylation sites in the linker region were mutated to aspartic acid (Fig. 2). In addition, as indicated, adjacent residues were also mutated to either glutamic or aspartic acid, and Tyr499 was mutated to Ala, based on evidence that this increases autophosphorylation of Rsk at Ser378 (28). To increase specific activity, the N-terminal 43 amino acids were truncated as previously described for CA-Rsk (4). The final construct, termed fully active Rsk (FA-Rsk) (Fig. 2) was tested for activity after expression in oocytes. mRNA encoding FLAG-tagged FA-Rsk was injected into oocytes, and entry into meiosis I (the G2/M transition) was monitored by scoring GVBD. As shown in Fig. 3A, in eight independent experiments, an average of 45% of the oocytes underwent GVBD in the absence of progesterone (range 0–90%). No GVBD was observed after expression of β-galactosidase, wild type Rsk (WT), or kinase-dead (N191A) WT or FA-Rsk. Western blotting experiments showed that all the active FLAG-tagged Rsk constructs were expressed at similar levels (Fig. 3B). Morphologically, GVBD induced by FA-Rsk

As an alternate approach, we undertook substitution of known activating phosphorylation sites in Rsk with acidic residues, either glutamic or aspartic acid. Previous studies with other kinases have shown that such substitutions can sometimes mimic the activating effects of phosphorylation and result in constitutive enzyme activity (20–22). In some cases, better constitutive activity has been obtained if residues adjacent to the phosphorylation site are also mutated to an acidic amino acid (23). For specific activity and GVBD activation assessments, constructs were expressed in resting and GVBD oocytes, respectively, immunopurified, and assessed for 56 kinase activity as described under “Materials and Methods.” Construct F was previously reported as CA-Rsk (4). Due to greater stability, construct E was chosen as the initial construct for further experiments in this paper.

![Structure/function analysis of Rsk1](http://www.jbc.org/)

Fig. 1. Structure/function analysis of Rsk1. Schematic representation of the eight deletion constructs, designated A–H, generated for these studies. A, wild type Rsk1. Three progressive carboxyl-terminal deletions eliminate first the carboxyl-terminal kinase domain (B), then the autophosphorylation site at Ser378 (C), and finally, the two MAPK sites at Thr568 (not shown, see Fig. 2) and Ser572 (D). These deletions were engineered independently (constructs B–D) or with an additional deletion of the first 43 amino acids of the amino terminus (constructs E–H). For specific activity and GVBD activation assessments, constructs were expressed in resting and GVBD oocytes, respectively, immunopurified, and assessed for 56 kinase activity as described under “Materials and Methods.” Construct F was previously reported as CA-Rsk (4). Due to greater stability, construct E was chosen as the initial construct for further experiments in this paper.

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was indistinguishable from that induced by progesterone. Moreover, in FA-Rsk expressing oocytes that underwent GVBD either with (G) or without (S) progesterone treatment, MAPK became activated and cyclin B2 was shifted electrophoretically, indicative of M phase, as also shown by Tyr15 dephosphorylation of cyclin B/Cdc2 (Fig. 3B). These biochemical features were equivalent to those observed with progesterone-treated control oocytes. Other studies with Rsk produced in Si9 cells show that after purification FA-Rsk has a specific activity five times greater than wild type Rsk (data not shown).

Assays of the total cellular activity of the FLAG-tagged FA-Rsk showed substantial activity in a G2 environment, ~40% of the level seen in M phase with wild type enzyme (Fig. 3C, lanes 2 and 4). Activity at GVBD in those oocytes that matured in response to FA-Rsk (S, lane 5 in Fig. 3C) was even higher and equivalent to progesterone-treated controls (compare lanes 2 and 5), due in part to increased translation and accumulation of FA-Rsk in the 5 h required for GVBD. To directly compare WT Rsk with FA-Rsk, the specific activity of the constructs was determined as described under “Materials and Methods” (Fig. 3D). It is evident that FA-Rsk achieved in a G2 environment nearly the same specific activity as wild type Rsk in M phase (Fig. 3D, compare lanes 2 and 4). The specific activity of FA-Rsk at GVBD was even higher and was the same in the presence or absence of progesterone.

As described in the Introduction, CA-Rsk is able to partially inhibit the APC and stimulate cyclin B synthesis even in the presence of U0126 (8), which completely inhibits the endogenous MAPK/Rsk pathway (4, 8, 18). As shown in Fig. 3B, GVBD induced by FA-Rsk led to activation of the endogenous MAPK pathway, most likely due to an established feedback loop from active Cdc2 to translational activation of Mos mRNA (29, 30). To investigate whether the ability of FA-Rsk to cause GVBD is dependent on an active endogenous MAPK pathway, FA-Rsk was expressed by mRNA injection into oocytes incubated in U0126 to inhibit the MAPK pathway (Fig. 4). In eight independent experiments, an average of 26% of oocytes still underwent GVBD in the absence of endogenous MAPK activity.

Biochemical analysis (Fig. 4B) confirmed equivalent action of FA-Rsk on cyclin B2/Cdc2 (MPF) activation regardless of MAPK activation. Total FA-Rsk activity in the oocyte was higher at GVBD in the absence of U0126 (Fig. 4C), but this difference reflected higher expression of FA-Rsk in the absence of U0126, as judged by FA-Rsk specific activity (Fig. 4D). Higher expression in the absence of U0126 is not unexpected in light of reports of increased protein synthesis after MAPK activation. Total FA-Rsk activity in the oocyte was significantly higher at GVBD. Extracts were prepared from resting oocytes (R), progesterone-treated oocytes that underwent GVBD (G), FA-Rsk expressing oocytes that underwent GVBD without progesterone (S), and oocytes injected with kinase-dead Rsk (NA). Lysates of these samples were blotted for the FLAG epitope, phosphorylated (active) MAPK (pMAPK), Cyclin B2, and phosphorylated (inactive) Cdc2 (pCdc2). Progesterone treatment. C, the protein products of constructs were immunoprecipitated from aliquots of the indicated extracts immunoblotted in B and assessed for S6 kinase activity as described under “Materials and Methods.” In addition to activity assays, these same immunoprecipitates were also immunoblotted for the FLAG epitope to evaluate expression levels (lower panel). D, total activities of the constructs shown in C were normalized to their respective signals on the FLAG immunoblot using densitometry as described under “Materials and Methods” to give specific activity.

DISCUSSION

The results in this paper report the generation of a form of Xenopus Rsk 1 that is constitutively active both in M phase and in the resting G2 phase of an immature oocyte. Constitutive activity was defined previously as activity that is not depend-
activity as well as in the presence of U0126, which blocks all detectable MAPK activation. The multiple negative charges at each of the MAPK phosphorylation sites in the linker proved to be important for generation of constitutive activity in a G2 environment inasmuch as single acidic mutations at the phosphorylation sites did not result in constitutive activity (Ref. 24 and data not shown).

The basis for higher constitutive activity of FA-Rsk in G2 than was evident for CA-Rsk may involve enhanced phosphorylation of Ser220 by PDK-1. Replacement of Ser220 with multiple acidic residues resulted in forms that lacked any detectable activity toward S6 peptide, and mutation of Ser378, which forms a docking site for PDK-1 (19, 27), also did not result in an active enzyme. Finally, maximal constitutive activity was only observed after mutation of Thr409 to alanine (data not shown), a change reported to increase phosphorylation at Ser378 (28). These considerations point to Ser220 phosphorylation by PDK-1 as a key event in activation. It was suggested previously for CA-Rsk (4) that PDK-1 activity may be higher in M phase than in G2, or binding to Rsk at Ser378 may increase, possibly accounting for higher FA-Rsk activity in M phase. However, with FA-Rsk the specific activity in G2 and M phases is similar even in the presence of U0126 (Fig. 4). The lower total activity of FA-Rsk in G2 phase oocytes appears to follow directly from lower expression levels (Figs. 3 and 4). Expression may be higher in oocytes with GVBD due to a general increase in protein synthesis rate after GVBD (1, 2). The constitutive activity of FA-Rsk in G2 phase allowed an evaluation of the role of Rsk in mediating MAPK effects on entry into meiosis. Approximately 45% of oocytes underwent GVBD in response to FA-Rsk expression, even though total oocyte Rsk activity in G2 was only 40% of that present at M phase with wild type enzyme (Fig. 3). However, the specific activity in G2 was equivalent to that of M phase Rsk; this could suggest that Rsk exists in a complex with substrates or regulators so that specific activity is more important than total activity. In support of this concept, Rsk is known to exist in a complex with MAPK and/or Myt 1 in oocytes (31, 32), and CSF arrest in embryonic blastomeres by CA-Rsk is evident even when total Rsk activity is below that present in CSF-arrested unfertilized eggs (4). A substantial response to FA-Rsk was still observed in the presence of U0126, which blocks even basal activity of the MAPK pathway. Previously we reported that a constitutively active form of MAPK could induce GVBD in oocytes (13). The present results indicate that this effect of MAPK is directly mediated by p90Rsk, and combined with previous reports (4, 5, 8), it appears that at all stages of maturation the effects of the MAPK pathway are mediated solely by activation of Rsk. It is notable that not all oocytes underwent GVBD with FA-Rsk, but even those that did not reach GVBD nevertheless were greatly accelerated into the G2/M transition upon subsequent progesterone administration. This indicates that other pathways besides MAPK are also likely to be important for the G2/M transition. In particular, the polo-like kinase pathway has been implicated in the activation of the phosphatase Cdc25C, which is required for the dephosphorylation and activation of cyclin B/Cdc2 (MPF) (1, 2). The relative insensitivity of Cdc25C activation to the MAPK pathway has been seen previously. Mos, a MAPKKK, and MAPKK induce maturation very slowly when expressed (15, 16). Thio-phosphorylated MAPK also induces maturation very slowly (16) and does not result in complete Cdc25C activation. Finally, Mos addition to oocyte extracts activates MAPK but does not activate Cdc25C (33, 34). In contrast, immunodepletion of Ptk1 from oocyte extracts cycling through maturation completely blocks Cdc25C activation even when the MAPK pathway is fully activated by Mos (34).
Rsk Promotes the G2/M Transition

In any case, while the results here suggest the effects of MAPK on the G2/M transition are mediated by Rsk, the substrate for Rsk involved in this transition is not clear at present. The original substrate for Rsk in this system, ribosomal protein S6, seems unlikely to be involved in cell cycle progression. In M phase, the actions of Rsk to inhibit the APC have been linked to the ability of Rsk to phosphorylate and activate the protein kinase Bub1 (6), a key regulator of the APC and spindle assembly checkpoint (7). However, Bub1 is inactive until G1VB (6) and therefore is unlikely to be important for entry into M phase. Another protein kinase, glycogen-synthase kinase 3 (GSK-3) can be inactivated by phosphorylation at Ser9 by Rsk, and therefore is unlikely to be important for entry into M phase, the actions of Rsk to inhibit the APC have been linked to the original substrate for Rsk involved in this transition is not clear at present.

The specific kinase responsible for inactivation of GSK-3 during MPF in resting G2 phase oocytes by phosphorylating Tyr15 and Thr14 in the Cdc2 ATP-binding site (32). The level of tyrosine phosphorylation is known to be rate-limiting for MPF activation (1, 2). Rsk is able to phosphorylate and inactivate Myt1 in vitro (32), promoting the dephosphorylation and activation of MPF, leading to GVBD. Support for this model comes from evidence that CA-Rak causes phosphorylation of Myt1 in the presence of U0126 at the meiosis I → II transition (8).

Recent evidence in several systems has suggested that nuclear progesterone receptors can activate MAPK by a nongenomic mechanism, most likely by interaction with the SH3 domain of pp60c-src and other signaling molecules (37, 38). It has been reported that overexpression of the nuclear progesterone receptor in Xenopus oocytes accelerates progesterone-induced GVBD (39, 40), and this acceleration is abolished by mutations in a proline-rich sequence in the receptor that binds SH3 domains (38). Microinjection of active pp60c-src into oocytes also accelerates GVBD and increases S6 phosphorylation due to Rsk (41). The ability shown here of FA-Rsk to induce GVBD by itself or to accelerate the rate of progesterone-induced GVBD suggests that Rsk is also the mediator of nongenomic signaling through the MAPK pathway by nuclear progesterone receptors in oocytes.

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