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tpr-met Oncogene Product Induces Maturation-Producing Factor Activation in *Xenopus* Oocytes

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***tpr-met*, a tyrosine kinase oncogene, is the activated form of the *met* proto-oncogene that encodes the receptor for hepatocyte growth factor/scatter factor. The *tpr-met* product ($p65^{tpr\text{-}met}$) was tested for its ability to induce meiotic maturation in *Xenopus* oocytes. While *src* and *abl* tyrosine kinase oncogene products have previously been shown to be inactive in this assay, $p65^{tpr\text{-}met}$ efficiently induced maturation-promoting factor (MPF) activation and germinal vesicle breakdown (GVBD) together with the associated increase in ribosomal S6 subunit phosphorylation. *tpr-met*-mediated MPF activation and GVBD was dependent on the endogenous c-mos^{xe}, while the increase in S6 protein phosphorylation was not significantly affected by the loss of *mos* function. The phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine inhibits *tpr-met*-mediated GVBD at concentrations that prevent insulin- but not progesterone-induced oocyte maturation. Moreover, maturation triggered by *tpr-met* is also inhibited by cyclic AMP-dependent protein kinase. This is the first demonstration that a tyrosine kinase oncogene product, $p65^{tpr\text{-}met}$, can induce meiotic maturation in *Xenopus* oocytes and activate MPF through a *mos*-dependent pathway, possibly the insulin or insulinlike growth factor 1 pathway.**

The c-*met* proto-oncogene product has recently been identified as the hepatocyte growth factor/scatter factor receptor (7, 27). The *met* proto-oncogene was discovered via the activated oncogene, *tpr-met* (46), that was generated in a human osteogenic cell line treated with *N*-methyl-*N*-nitro-*N*-nitrosoguanidine (11). Activation resulted from a DNA rearrangement between the *tpr* sequence from chromosome 1 and downstream *met* tyrosine kinase receptor sequences located on chromosome 7 (28, 46). The *tpr-met* product, $p65^{tpr\text{-}met}$, induces transformation of NIH 3T3 cells as a constitutively expressed tyrosine kinase (11).

The *Xenopus* oocyte system is useful for examining the events involved in signal transduction (58). Fully grown *Xenopus* oocytes arrested at the G_2/M border (40) can be induced to enter M phase by progesterone, insulin, or insulinlike growth factor 1 (IGF-1). A marked decrease in cyclic AMP (cAMP) levels is an early biochemical change that occurs in oocytes after progesterone treatment. This decrease is due to inhibition of adenylate cyclase (22, 31, 49–51). In contrast, maturation induced by insulin and IGF-1 involves inhibition of adenylate cyclase as well as stimulation of a phosphodiesterase activity (52, 53). The hormones initiate the activation of maturation-promoting factor (MPF), germinal vesicle breakdown (GVBD), chromosome condensation, completion of meiosis I, and progression to metaphase arrest at meiosis II. MPF is a cytoplasmic protein kinase activity that participates in GVBD as well as chromosome condensation (38, 42) and consists of the homologs for the major cell cycle oscillator $p34^{cdc2}$ and B-type cyclins (15, 17, 24, 25, 41, 47). The *mos* proto-oncogene product is required for both progesterone- and insulin-induced oocyte maturation and therefore must function downstream from where these pathways intersect (56). The *mos* product has been shown to be required throughout meiotic maturation (13, 33) and is an active component of cytostatic factor (57). Cytostatic factor is believed to be responsible for the arrest of mature oocytes at metaphase II (40) by stabilizing MPF (57). Protein hyperphosphorylation occurs during meiotic maturation in *Xenopus* oocytes (39). The 40S ribosomal subunit S6 (20) is one growth-associated protein that is hyperphosphorylated during oocyte maturation (34, 44), and both S6 kinase phosphorylation and S6 protein phosphorylation increase in *Xenopus* oocytes after progesterone or insulin treatment (18, 19, 37, 61).

The *ras* oncogene (2–4, 12, 14) and *mos* proto-oncogene (23, 55) products induce progesterone-independent meiotic maturation in *Xenopus* oocytes, while v-*src* (59) and v-*abl* (37) tyrosine kinase oncoproteins do not. However, expression of exogenously added epidermal growth factor receptor or the *trk* proto-oncogene product followed by treatment with the respective ligands (epidermal or nerve growth factor) induces oocyte maturation (43, 45). Thus, receptor tyrosine kinases can initiate meiotic maturation, but the pathway(s) that they utilize to influence G_2/M transition and MPF activation is not known. Receptor tyrosine kinases have been shown to activate MPF in oocytes, while intracellular oncogenic tyrosine kinases have not. We have studied the influence of an oncogenic form of a receptor tyrosine kinase, $p65^{tpr\text{-}met}$, on *Xenopus* oocyte maturation. This system was used as a tool to determine possible pathways involved in MPF activation by oncogenic receptor kinases and how these pathways may differ from those affected by the nonreceptor tyrosine kinase oncoproteins. We show that the *tpr-met* oncogene can induce GVBD and activate meiotic maturation in the absence of hormone stimulation. To begin to identify the activation pathway, we have tested whether phosphodiesterase inhibitors or the cAMP-dependent protein kinase catalytic subunit alters the ability of $p65^{tpr\text{-}met}$ to induce maturation events. We have

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also determined the influence of *tpr-met* on S6 protein phosphorylation.

MATERIALS AND METHODS

Frogs and oocytes. *Xenopus laevis* females were purchased from *Xenopus* I (Ann Arbor, Mich.). Oocytes were surgically removed and defolliculated either manually or by incubation in modified Barth solution [MBS; 88 mM NaCl, 1 mM KCl, 2.5 mM $NaHCO_3$, 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.5), 0.82 mM $MgSO_4$, 0.33 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$] containing collagenase A (1.5 mg/ml; Boehringer Mannheim Biochemicals, Indianapolis, Ind.) for 2 h (29). The oocytes were washed extensively, and stage VI oocytes (16) were isolated and maintained overnight in either MBS or 50% L15 media (GIBCO, Grand Island, N.Y.).

Oligodeoxyribonucleotide and RNA injections. Eighteen hours after oocyte isolation, microinjections were performed by using an Attocyte injector (ATTO Instruments, Rockville, Md.) with 40 nl of a mixture of either antisense or sense *mos*-specific oligonucleotides (3 mg/ml) designated A±, B±, C±, and D± as described by Sagata et al. (56). Subsequent injection of in vitro-synthesized *tpr-met* transcripts was performed 4 h later. The cDNA encoding the *tpr-met* oncogene product (46) had been inserted into the *Eco*RI restriction site of the Bluescript SK vector (Stratagene, La Jolla, Calif.). The v-*src* DNA was inserted into the *Nde*I restriction site (containing the translational initiator codon for the T7 capsid gene) of the T7 pAR vector. Capped RNA was synthesized by using T7 RNA polymerase and reaction conditions specified by the vender (Promega, Madison, Wis.). The protein kinase A (PKA) catalytic subunit cDNA (TPK-1; gift from M. Wigler) was engineered into a T7 RNA polymerase-containing vector as described by Johnson et al. (30) and transcribed as stated above. Oocytes were scored for GVBD, as evidenced by the appearance of a white spot at the animal pole. This observation was verified by manual dissection of oocytes after fixation in 10% trichloroacetic acid.

S6 phosphorylation. Stage VI oocytes were isolated and prelabeled for 3.5 h at 20°C in MBS containing $^{32}P_i$ (0.3 mCi/ml; Amersham, Arlington Heights, Ill.). Oocytes were injected with either *tpr-met* RNA (10 ng per oocyte) or buffer and placed in fresh MBS. Groups of 20 injected oocytes were harvested over a 9-h period and homogenized in 1 ml of lysing buffer [50 mM piperazine-*N*,*N'*-bis(2-ethanesulfonic acid (PIPES; pH 7.5), 5 mM $MgCl_2$, 5 mM KCl, 50 mM NaF, 4 μM EDTA, 1% deoxycholate, 1% Triton X-100]. Extracts were clarified by centrifugation at 10,000 × *g* for 10 min at 4°C. The supernatant was layered over 2 ml of buffer containing 1.6 M sucrose, 50 mM PIPES (pH 7.5), 5 mM $MgCl_2$, 0.5 mKCl, and 4 μM EDTA and centrifuged at 100,000 × *g* for 16 h at 4°C. The ribosome fraction pellets were suspended in 2× sample buffer and analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) on a 10% gel.

Phosphoamino acid analysis of S6 proteins. One hundred oocytes were ^{32}P labeled, and S6 proteins were analyzed as described above. The band representing the S6 protein was removed from the gel and homogenized in a buffer containing 0.5 M NH_4CO_3, 0.1% SDS, 5% β-mercaptoethanol, and 70 μg of bovine serum albumin. The homogenate was incubated at 37°C for 24 h; the gel fragments were then removed by centrifugation, and the S6 protein was precipitated in 20% trichloroacetic acid. The protein was pelleted at 15,000 × *g* for 10 min at 4°C and then washed two times with 100% ethanol and two times with an ether-ethanol (3:1) solution. The pellet was vacuum dried and hydrolyzed by 6 N HCl for 1.5 h at 110°C. The phosphoamino acids were separated by thin-layer chromatography and visualized by autoradiography.

MPF assay. Crude MPF extracts were prepared by homogenizing groups of 10 to 20 oocytes in 20 to 40 μl of extraction buffer (80 mM sodium β-glycerophosphate, 20 mM EGTA, 15 mM $MgCl_2$, 20 mM HEPES [pH 7.2], 1 mM ATP, 1 mM dithiothreitol 5 mM NaF). The homogenate was centrifuged at 16,000 × *g* for 5 min at 4°C, and the supernatant was used for microinjections. Groups of 12 to 14 oocytes were incubated in 1× MBS containing cycloheximide (10 μg/ml) for 1 h and then injected with 40 nl of the supernatant from each appropriate donor group. The recipient oocytes were cultured for 3 to 4 h in the presence of cycloheximide and then examined for GVBD.

Histone H1 kinase assay. Two microliters of extract was transferred into 50 μl of extraction buffer (80 mM sodium β-glycerophosphate, 20 mM EGTA, 50 mM $MgCl_2$, 20 mM HEPES [pH 7.2], 1 mM dithiothreitol, 2.5 mM phenymethysulfonyl fluoride, 10 μg of leupeptin per ml, 10 μg of aprotinin per ml, 10 μM protein kinase inhibitor). The histone H1 kinase reaction was performed by adding 10 μl of the sample to 6 μl of a mixture containing 1 μg of histone H1, 1 mM ATP, and 1 μCi of [γ-^{32}P]ATP. The reaction mixture was incubated at room temperature for 15 min, and then the reaction was stopped by the addition of an equal volume of 2× sample buffer (4% SDS, 20% glycerol, 150 mM Tris-HCl [pH 6.8], 0.02% bromophenol blue, 5% β-mercaptoethanol). Samples were resolved by SDS-PAGE on a 10% gel. Gels were fixed in 40% methanol–5% acetic acid, dried, and exposed to film.

RESULTS

To test the effect of *tpr-met* on oocyte maturation, in vitro-transcribed RNA was injected into fully grown stage VI *Xenopus* oocytes. RNA encoding the $pp60^{v\text{-}src}$ oncogene product was used as a control. Oocytes injected with RNA encoding the *tpr-met* oncoprotein expressed $p65^{tpr\text{-}met}$ (data not shown), and *tpr-met* transcripts induced GVBD in a dose-dependent manner (Fig. 1). One to five nanograms of *tpr-met* RNA was sufficient to induce >90% GVBD in 18 h. Moreover, cytosolic extracts prepared from these oocytes were positive for MPF, demonstrating that the eggs were arrested at metaphase. As previously reported (59), v-*src* was negative in this assay (Fig. 1). To determine whether the endogenous c-*mos* was required for *tpr-met*-induced GVBD, c-mos^{xe}-specific antisense or sense oligonucleotides (55) were injected into oocytes 3.5 h prior to injection of *tpr-met* RNA. Only the c-mos^{xe} antisense oligonucleotides prevented *tpr-met*-induced GVBD and to the same extent as in progesterone-dependent maturation (3, 12, 56) (Fig. 2). Thus, the *tpr-met* tyrosine kinase product can induce meiotic maturation in a *mos*-dependent manner and must therefore function upstream of the *mos* product.

We determined whether *tpr-met* induced the phosphorylation of the ribosomal subunit S6 since this is an early event in insulin- or progesterone-stimulated oocyte maturation (44). Fully grown stage VI oocytes were prelabeled with $^{32}P_i$ for 3.5 h (32) and subsequently injected with 10 ng of *tpr-met* RNA. Over a period of 9 h, ribosomes were isolated and phosphoproteins were analyzed by SDS-PAGE (Fig. 3). S6 protein phosphorylation increased between 3 and 9 h postin-

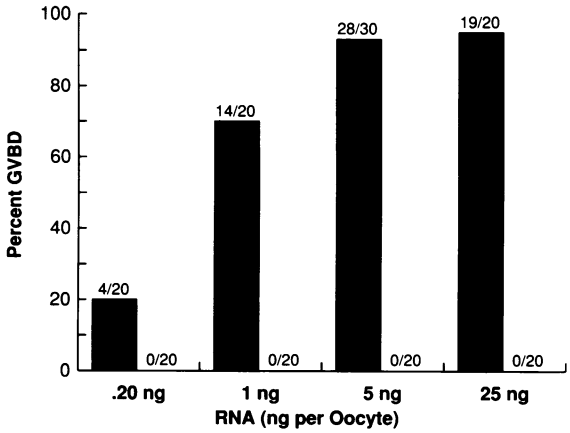

FIG. 1. Induction of oocyte maturation by *tpr-met* RNA. Fully grown oocytes were microinjected with 0.2 to 25 ng of capped *tpr-met* transcripts (black bars) or capped v-*src* transcripts (white bars). The percentage of oocytes undergoing GVBD is represented by the histogram bars, and the number of oocytes with GVBD over the number injected is displayed above each bar.

jection, but the major increase occurred at a time equivalent to its appearance after progesterone- or insulin-induced maturation, that is, at 0.5 to 0.6 $GVBD_{50}$ (Fig. 3). Progesterone- and insulin-induced S6 protein phosphorylation has been shown to require the endogenous *mos* product (3). We therefore expected that *tpr-met*-induced S6 phosphorylation would also be blocked in the presence of antisense c-mos^{xe} oligonucleotides. Oocytes were prelabeled with $^{32}P_i$ and preinjected with either sense or antisense *mos* oligonucleotides 3 h before *tpr-met* RNA was injected. As previously shown, only oocytes injected with *tpr-met* RNA and c-mos^{xe} sense oligonucleotides display GVBD. However, very similar levels of S6 protein phosphorylation were observed in both sense and antisense c-mos^{xe} oligonucleotide-injected oocytes (Fig. 4A). Thus, *tpr-met* stimulates S6 protein

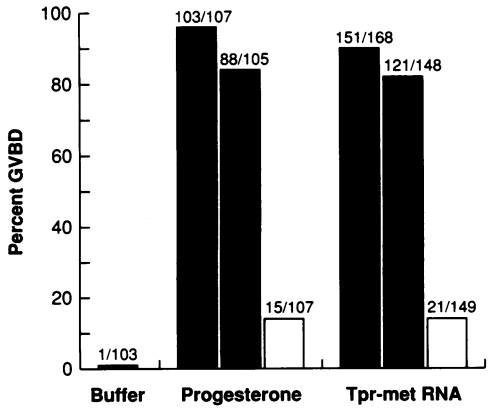

FIG. 2. Inhibition of *tpr-met*-induced GVBD by *mos* antisense oligonucleotides. Stage VI oocytes were injected with either *mos* antisense (white bars) or sense (gray bars) oligonucleotides 3.5 to 4 h before the injection of capped *tpr-met* transcripts or progesterone (10 μM) treatment. The oocytes were examined 18 h later for GVBD. The percentages of oocytes undergoing GVBD as a result of progesterone treatment or injection of *tpr-met* RNA or buffer alone are represented by black bars. The number of oocytes that underwent GVBD over the number injected or treated with hormone is displayed above each bar.

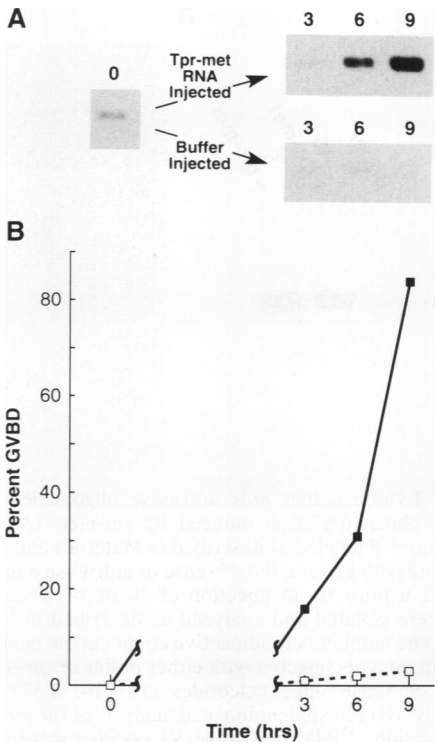

FIG. 3. Phosphorylation of S6 protein in oocytes after microinjection of *tpr-met* RNA (10 ng). (A) ^{32}P-labeled stage VI oocytes were injected with *tpr-met* RNA or buffer. Oocytes were harvested over the indicated period of time after injection, and phosphorylated S6 proteins in ribosomes were analyzed by SDS-PAGE and autoradiography. (B) Maturation of oocytes induced by *tpr-met* RNA (closed squares) was determined for the same batch of oocytes. Oocytes injected with buffer are represented by open squares. The radioactivities in bands 0, 3, 6, and 9 of buffer injected are 384, 254, 254, and 234 cpm, respectively, and those in band 3, 6, and 9 of *tpr-met* injected are 306, 827, and 1,994 cpm, respectively.

phosphorylation in the absence of $pp39^{mos}$. Phosphoamino acid analysis of the S6 protein showed only phosphoserine residues (Fig. 4B), and hyperphosphorylation was therefore not the result of direct tyrosine phosphorylation by $p65^{tpr\text{-}met}$.

Progesterone stimulation of oocytes has been reported to result in the decrease of cAMP as well as the cAMP-dependent PKA activity (35, 58), and injection of the catalytic PKA subunit into *Xenopus* oocytes blocks progesterone-induced meiotic maturation (38, 58). We tested the influence of PKA activity on *tpr-met* function by coinjecting fully grown oocytes with RNA encoding the PKA catalytic subunit and *tpr-met*. As with progesterone (38), the catalytic PKA subunit also prevented *tpr-met* induction of meiotic maturation (Fig. 5), even when *tpr-met* transcripts were injected 1 h before PKA RNA (data not shown). These experiments raised the question of whether *tpr-met*-induced maturation was sensitive to 3-isobutyl-1-methylxanthine (IBMX). IBMX, a phosphodiesterase inhibitor, has been shown to be a more potent inhibitor of the insulin-induced oocyte maturation pathway than it is of progesterone-induced maturation (53). We determined whether *tpr-met*-induced oocyte maturation was also sensitive to low levels of IBMX. Oocytes were injected with *tpr-met* RNA and exposed to the IBMX at concentrations that inhibit insulin- and

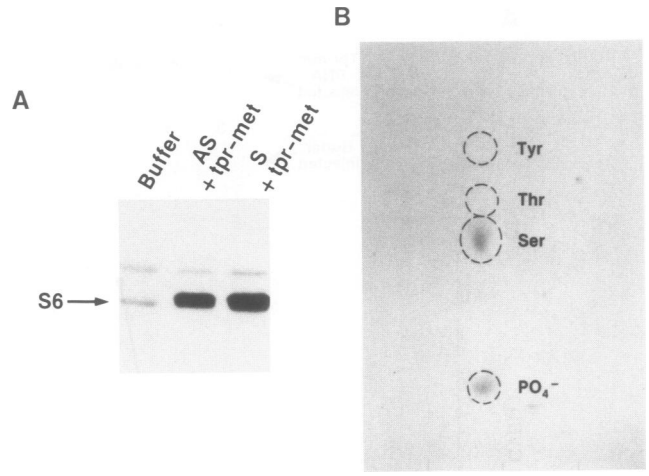

FIG. 4. Evidence that *mos* antisense oligonucleotides do not inhibit S6 phosphorylation induced by *tpr-met*. (A) Twenty-five oocytes were ^{32}P labeled as described in Materials and Methods and then injected with either c-*mos*^{xe} sense or antisense oligonucleotides or buffer 2 h prior to an injection of 10 ng of *tpr-met* RNA. S6 proteins were isolated and analyzed as described in Materials and Methods. The numbers of radioactive counts in the bands representing S6 from oocytes injected with either buffer or *tpr-met* RNA and antisense or sense oligonucleotides are 614, 2,377, and 3,355, respectively. (B) Phosphoamino acid analysis of *tpr-met*-induced S6 phosphorylation. ^{32}P-labeled stage VI oocytes were injected with *tpr-met* RNA. Oocytes were harvested 18 h later, and S6 proteins were fractionated by SDS-PAGE. The S6 protein was then subjected to phosphoamino acid analysis as described in Materials and Methods. The phosphorylated amino acids were visualized by autoradiography.

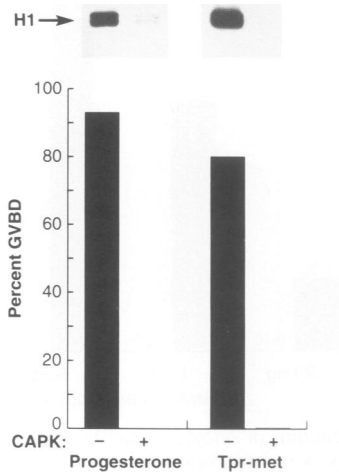

FIG. 5. Inhibition of *tpr-met*-induced maturation by expression of the PKA catalytic subunit. Stage VI oocytes were treated with progesterone (10 μM) or injected with capped *tpr-met* RNA (10 ng) at the same time they either were (+) or were not (−) being injected with the PKA catalytic subunit RNA (CAPK). GVBD was assessed 18 h later. A subset of 10 oocytes was harvested, and homogenates were prepared and tested for histone H1 kinase activity as described in Materials and Methods. The phosphorylated histones were analyzed by 10% SDS-PAGE and autoradiography. The resulting bands are displayed above the corresponding histogram bars.

IGF-1 (20 to 50 μM)- but not progesterone-induced meiotic maturation (53) (Fig. 6). These analyses show that IBMX treatment prevents *tpr-met*-induced GVBD and the appearance of MPF-associated H1 histone kinase activity (Fig. 6), suggesting that the oncogene functions through the insulin or IGF-1 pathway.

DISCUSSION

tpr-met is the first tyrosine kinase oncogene product shown to induce meiotic maturation in *Xenopus* oocytes. Sadler and Maller have used nonselective phosphodiesterase inhibitors (IBMX, papaverine, and theophylline) to show that oocyte maturation induced by insulin, IGF-1, and oncogenic *ras* protein requires the stimulation of a phosphodiesterase, while maturation induced by progesterone does not (53). Our studies show that *tpr-met*-induced maturation is sensitive to IBMX at concentrations equivalent to those that inhibit insulin, IGF-1, and the *ras* oncoprotein (53), suggesting that *tpr-met* also functions in this pathway and not the progesterone pathway. Although uncertain, it is possible that the *tpr-met* product as well as certain tyrosine kinase growth factor receptors such as those for insulin, IGF-1, epidermal growth factor, and nerve growth factor induce oocyte maturation through a similar pathway that converges with the natural progesterone pathway upstream from *mos*.

It has been suggested that low concentrations of IBMX (30 μM) inhibit insulin-induced maturation by arresting cAMP-phosphodiesterase activity and, there is a large body of data showing that induction of oocyte maturation results in a decrease of intracellular cAMP (1, 35, 38, 54). There are exceptions, however; Birchmeier et al. (4) and Sadler et al. (54) have both reported that cAMP levels are not reduced during meiotic maturation triggered by the p21^{ras} oncoprotein. Moreover, Gelerstein et al. (26) showed that acetylcholine treatment of oocytes lowers cAMP levels but does not initiate GVBD (26). They also show that adenosine prevents cAMP reduction in oocytes that are induced to mature with progesterone (26). Despite these discordant results, it has been shown that the cAMP-dependent PKA catalytic subunit inhibits progesterone-induced maturation (38). We show that the PKA catalytic subunit inhibits *tpr-met*-induced maturation, suggesting that the PKA acts downstream from the point in the maturation pathway where progesterone and *tpr-met* converge.

In *Xenopus* oocytes, the ribosomal S6 protein has been shown to be hyperphosphorylated after initiation of meiotic maturation by progesterone (19, 44), insulin (18, 36), or the activated *ras* oncoprotein (3, 32). However, S6 phosphorylation is also stimulated by v-*src* (59) and v-*abl* (37) protein kinases, even though these products are unable to induce meiotic maturation. The *tpr-met* product also induces phosphorylation of ribosomal S6 protein, even when maturation is prevented by loss of endogenous pp39^{mos}, and appears to be similar to the *ras* oncoprotein in this regard (3). Ribosomal S6 protein phosphorylation is implicated in cell proliferation and transformation (5, 6, 35, 59, 61), and results from many laboratories indicate that in *Xenopus* oocytes as well as in cultured cells, there are multiple pathways that lead to S6 phosphorylation.

S6 kinases are normally activated during *Xenopus* oocyte maturation (19) and can be activated by murine mitogen-activated protein kinases in vitro (17). Mitogen-activated protein kinases resemble extracellular signal-related kinases (8, 48) and myelin basic protein kinases (9, 48), which become tyrosine phosphorylated as MPF is activated in *Xenopus* oocytes (10, 21), and these kinases may activate S6

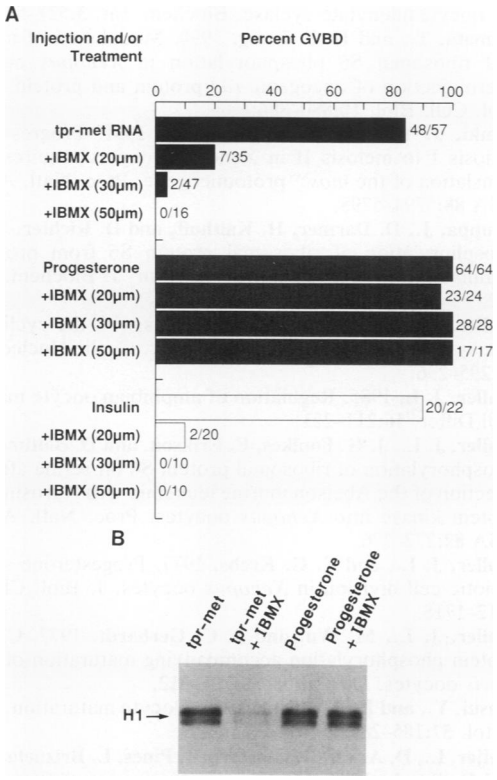

FIG. 6. Inhibition by IBMX of *tpr-met* RNA-induced GVBD and histone H1 kinase activity. (A) Fully grown stage VI oocytes were injected with capped *tpr-met* RNA (10 ng) or treated with progesterone (10 μM) or insulin (2 μM) in the presence of 0 to 50 μM IBMX as indicated. Oocytes were examined for GVBD 9 to 18 h later. The number of oocytes that underwent GVBD over the number injected or treated with hormone is represented at the end of the appropriate histogram bar. (B) Fully grown stage VI oocytes were injected with capped *tpr-met* RNA or treated with progesterone (10 μM) in the presence or absence of 30 μM IBMX; 10 to 20 oocytes were collected 9 h later, and homogenates were prepared and tested for histone H1 kinase activity as described in Materials and Methods. The phosphorylated histones were analyzed by 10% SDS-PAGE and autoradiography.

kinases (9, 10, 20, 21). It is also possible that *tpr-met* and some other oncoproteins (v-*src* and *ras*) activate S6 protein kinase(s) or an S6 protein kinase activator(s) present in *Xenopus* oocytes that does not require *mos* function or alternatively, and they may inactivate an S6 protein phosphatase.

Another possibility may be that in oocytes, polysomes are preprogrammed with specific maternal mRNA to function at different stages of maturation or early development and that their activation occurs in conjunction with S6 phosphorylation. For example, pp39^{mos} synthesis occurs within 1 to 2 h after progesterone treatment (55), and perhaps pp39^{mos} synthesis requires ribosomal subunit S6 phosphorylation to initiate translation from *mos*-specific polysomes. In this model, other inducers of S6 phosphorylation (v-*src* and v-*abl*) may target different polysome fractions of the developmental program. Further, our data with *tpr-met* are similar to the S6 phosphorylation results obtained by Barrett et al. (3) with the *ras* oncoprotein in *mos*-depleted oocytes. Interestingly, they show that S6 phosphorylation induced by insulin and progesterone is inhibited by *mos* depletion (3). This would indicate that S6 hyperphosphorylation is downstream of *mos* or alternatively that S6 phosphorylation of *mos*-specific polysomes is a key early event. In this model, the slight reduction in S6 phosphorylation observed when either the *ras* oncoprotein (3) or *tpr-met* (Fig. 4A) is introduced into *mos*-depleted oocytes may result from reduction of the phosphorylation of endogenous *mos*-specific polysome required for the translation of *mos* product and the initiation of the maturation process. In this model, it would also be possible for *tpr-met* to function through an S6 phosphorylation-dependent feedback loop, initiating *mos* translation and thereby causing entry into the maturation pathway.

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