Requirement for Raf and MAP Kinase Function during the Meiotic Maturation of *Xenopus* Oocytes

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Abstract. The role of Raf and MAPK (mitogen-activated protein kinase) during the maturation of *Xenopus* oocytes was investigated. Treatment of oocytes with progesterone resulted in a shift in the electrophoretic mobility of Raf at the onset of germinal vesicle breakdown (GVBD), which was coincident with the activation of MAPK. Expression of a kinase-defective mutant of the human Raf-1 protein (KD-RAF) inhibited progesterone-mediated MAPK activation. MAPK activation was also inhibited by KD-Raf in oocytes expressing signal transducers of the receptor tyrosine kinase (RTK) pathway, including an activated tyrosine kinase (Tpr–Met), a receptor tyrosine kinase (EGFr), and Ha-Ras^{V12}. KD-RAF completely inhibited GVBD induced by the RTK pathway. In contrast, KD-RAF did not inhibit GVBD and the progression to Meiosis II in progesterone-treated oocytes. In-jection of Mos-specific antisense oligodeoxy-ribonucleotides inhibited MAPK activation in response to progesterone and Tpr–Met, but failed to inhibit these events in oocytes expressing an oncogenic deletion mutant of Raf-1 (ΔN'Raf). Injection of antisense oligodeoxyribonucleotides to Mos also reduced the progesterone- and Tpr–Met-induced electrophoretic mobility shift of *Xenopus* Raf. These results demonstrate that RTKs and progesterone participate in distinct yet overlapping signaling pathways resulting in the activation of maturation or M-phase promoting factor (MPF). Maturation induced by the RTK pathway requires activation of Raf and MAPK, while progesterone-induced maturation does not. Furthermore, the activation of MAPK in oocytes appears to require the expression of Mos.

T HE c-*raf*-1 gene is the normal cellular counterpart of the v-*raf* transforming gene of the murine sarcoma virus 3611 (59). The proto-oncogene product encoded by the c-*raf*-1 gene, Raf-1, is a 70–74-kD phosphoprotein with intrinsic kinase activity towards serine and threonine residues (for review see Morrison [45]). The Raf-1 protein consists of a carboxyl-terminal kinase domain and an amino-terminal regulatory region that is deleted in v-*raf*, generating a constitutively active kinase (29, 68, 69). Raf-1 is ubiquitously expressed and is hyperphosphorylated, primarily on serine residues, in many cell lines in response to mitogen treatment (30, 46). A close correlation has been established between Raf-1 hyperphosphorylation and activation of its kinase activity in cells stimulated by growth factors (30, 40). Moreover, Raf-1 activity is required for growth factor-induced proliferation of NIH/3T3 cells and certain erythroid cells (11, 34).

Recently, genetic and biochemical evidence has placed Raf-1 in a signal transduction cascade downstream of both receptor tyrosine kinases (RTKs)[1] and ras, and upstream of

mitogen-activated protein kinase (MAPK) (for review see Roberts [60]). Furthermore, recent reports suggest that the protein which activates MAPK (also known as MAPK kinase [MAPKK]) is a substrate of Raf-1 both in vitro and in vivo (16, 32, 38). MAPK is activated in many mitogenically stimulated cell types (1–3, 7, 8, 31, 61) as well as in differentiating PC12 cells (9, 22, 24–26, 44, 70) and maturing *Xenopus* oocytes (4, 23, 27, 28, 53, 55). MAPK becomes phosphorylated on both threonine and tyrosine residues in response to activation of MAPKK, an event that correlates with an increase in its kinase activity (3, 4, 24, 36, 43, 56). The observation that MAPK is activated during processes as diverse as proliferation and differentiation of somatic cells as well as meiotic maturation of oocytes suggests that this protein has an important role in many signal transduction pathways.

Fully grown *Xenopus* oocytes are arrested in prophase of meiosis I, and are induced to mature upon exposure to progesterone (42). Progesterone stimulates the synthesis of

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the Mos protooncogene product, pp39^{mos}, which is required for the activation of maturation promoting factor (MPF), an activity responsible for coordinating the biochemical events of meiosis I and II (14, 33, 63, 64). During meiosis I, the rise in MPF activity is coincident with phosphorylation and activation of MAPK (23, 27, 55).

Insulin-like growth factor (IGF-1) is also capable of inducing maturation of *Xenopus* oocytes, through activation of its cognate RTK that is present on the surface of oocytes (21, 41). Similarly, maturation of oocytes has been induced by the expression and activation of the RTKs for EGF and NGF (trk) (48, 50), and by expression of an activated oncogenic form of the receptor for hepatocyte growth factor (Tpr-Met) (15). Although signal transduction by progesterone and RTKs results in the synthesis of Mos and the induction of MPF, the two pathways differ in several aspects. While maturation induced by Tpr-Met and IGF-1 requires the stimulation of a phosphodiesterase (15, 62), maturation induced by progesterone does not. Further, maturation-signaling cascades induced by IGF-1, but not those induced by progesterone, require the specific involvement of p21^{ras}, GAP, and PKC-ζ (5, 17, 19, 20, 35).

Since Raf plays an essential role in transducing proliferative signals in somatic cells and can induce MAPK activation, we examined the effects of a kinase-defective Raf protein (KD-Raf) on *Xenopus* oocyte maturation. In this report we show that microinjection of RNA encoding KD-Raf inhibits MAPK activation in oocytes stimulated by either progesterone or the injection of RNA encoding the tyrosine kinases Tpr-Met, epidermal growth factor receptor (EGFr), or oncogenic Ha-Ras^{V12}. We also demonstrate that injection of KD-Raf blocks the tyrosine kinase- and Ha-Ras^{V12}-induced GVBD, but does not prevent progesterone-stimulated maturation. Furthermore, injection of Mos-specific antisense oligonucleotides blocks MAPK activation promoted by either progesterone or Tpr-Met.

Materials and Methods

Frogs and Oocytes

Xenopus laevis females were purchased from *Xenopus* I (Ann Arbor, MI). Oocytes were removed and defolliculated by incubation in modified Barth solution (MBS; 88 mM NaCl, 1 mM KCl, 2.5 mM NaHCO₃, 10 mM Hepes, pH 7.5, 0.82 mM MgSO₄, 0.33 mM [CaNO₃]₂, 0.4 mM CaCl₂) containing collagenase A (1.5 mg/ml; Boehringer Mannheim Biochemicals, Indianapolis, IN) for 2 h. Oocytes were washed several times in MBS and cultured overnight in 50% Leibovitz-15 media (GIBCO BRL, Grand Island, NY).

cDNAs and RNA Transcription

The human c-*raf*-1 construct used for mutagenesis (pKS:cRaf) was created by filling in a 2.1-kb NdeI-XbaI fragment of the human c-*raf*-1 cDNA (encoding amino acids 1-648) with klenow, followed by ligation into the BamHI site of Bluescript KS by use of a linker. The KD-Raf mutant contains a serine to alanine substitution at position 621 (S621A) and was created by in vitro mutagenesis of pKS:cRaf using the oligonucleotide 5'-AAC-CGGAGCGCTGCCGAGCCATCC-3' and the procedure described by the vendor (Biorad Mutagenesis Kit, Richmond, CA). The resulting mutant construct (designated pKS:KD-Raf) was subsequently sequenced using a custom primer and the Sequenase Version 2.0 DNA Sequencing Kit (United States Biochemical Corp., Cleveland, OH) to confirm the specific base change. For the production of RNA in vitro, the coding regions of the wild-type and KD-Raf versions of the c-*raf*-1 gene were subcloned into the BglII site of the vector pSP64T (37). The ΔN'Raf construct, pKS:ΔN'Raf, con-

tains the coding region for amino acids 306-648 of the human Raf-1 kinase domain inserted in the EcoRV restriction site of the Bluescript KS vector (kindly provided by Martin McMahon of DNAX, Palo Alto, CA.). A 56-bp 5'-untranslated leader sequence from the *Xenopus* β-globin gene was added upstream of the ATG start site of pKS:ΔN'raf as a HindIII-NcoI fragment, resulting in the plasmid pGlo:ΔN'raf. The transcription vectors for Δ-N'Raf and Δ-KD-Raf were created by excising a 1.7-kb HindIII-BglII fragment (encoding amino acids 1-568) of pSP64T:Raf-1 and pSP64T:KD-Raf as and replacing them with an 0.8-kb HindIII-BglII fragment (encoding amino acids 306-568) from pGlo:ΔN'raf.

The cDNA encoding the Tpr-Met oncogene product (51) had been inserted into the EcoRI restriction site of the Bluescript SK vector (Stratagene, La Jolla, CA). Ha-ras^{V12} cDNA was inserted into SmaI-Bam HI restriction sites of the Sp65 vector (Promega, Madison, WI). The human EGF receptor, EGFr, cDNA (a gift of Lee Opresko and H. Steven Wiley, University of Utah Medical Center) was engineered into the SP64 polyA vector (50). All plasmid constructs were linearized with the appropriate restriction enzyme and capped RNA transcripts were synthesized as specified by the vendor (Ambion, Austin, TX) using either the T7 or Sp6 polymerases.

Injections

18 h after oocyte isolation, microinjections were performed using the attocyte injector (ATTO Instruments, Rockville, MD) with an injection volume of 30 nl. KD-Raf RNA transcripts (1.0 mg/ml) were microinjected 18-24 h before a subsequent injection of Tpr-Met or Ha-Ras^{V12} RNA. Experiments using the EGFr RNA were performed by microinjecting EGFr RNA (1.0 mg/ml), culturing the oocytes in 50% L-15 media with 5% FCS and 1% BSA for 24-30 h, and then microinjecting KD-Raf RNA (1.0 mg/ml) 4 h before adding EGF (0.1 μg/ml) to the medium. The experiments involving the EGFr were performed in this manner to ensure adequate translation of the EGFr. Mos-specific antisense or sense oligodeoxynucleotides (63) (90 ng per oocyte) were injected 1.5 h before injections of Tpr-Met RNA or the addition of progesterone (2 μg/ml). Oocytes were scored for germinal vesicle breakdown (GVBD) as evidenced by the appearance of a white spot at the animal pole. This observation was verified in many cases by manual dissection of oocytes after fixation in 8% TCA.

Western Blot Analysis

Oocytes were homogenized with 10 μl per oocyte of lysis buffer (137 mM NaCl, 20 mM Tris, pH 8.0, 2 mM EDTA, 1% NP-40) containing 1 mM PMSF, aprotinin (0.15 U/ml), 20 μM leupeptin, and 0.5 μM sodium vanadate. Insoluble material was removed by centrifugation at 14,000 g for 10 min at 4°C. Lysates were resolved by SDS-PAGE and electrophoretically transferred to 0.2 μM-pore nitrocellulose membranes (Schleicher & Schuell Inc., Keene, NH). Filters were blocked with 2% BSA (fraction 5; Sigma Immunochemicals, St. Louis, MO) in TBS (pH 8.0) for 1 h, washed three times in TBS containing 0.2% Tween 20 (TBST), and incubated overnight at 4°C with primary antibody diluted in TBST. For Western hybridization analysis a carboxy-terminal antipeptide Raf-1 antibody (65) and a MAPK-specific antibody (gift of John Blenis at Harvard Medical School) were used at dilutions of 1:2,000 and 1:5,000, respectively. Antibody against phosphotyrosine (4G10) was obtained from Upstate Biochemicals Incorporated (Lake Placid, NY) and was used according to the manufacturer's instructions. The filters were washed three times with TBST, incubated for 1 h with HRP-coupled secondary antibody (Boehringer-Mannheim Corp.) diluted 1:20,000 in TBST, washed with TBST, and developed using enhanced chemiluminescence (Amersham Corp., Arlington Heights, IL). Data from Western blots were quantified using an LKB UltraScan XL densitometer.

Myelin Basic Protein In Vitro Kinase Assay

Immunoprecipitations were performed by incubating oocyte lysates with a MAPK specific antibody (74) for 3 h at 4°C. Protein A-Sepharose beads were used to collect the antigen-antibody complexes which were then washed three times with lysis buffer plus 0.5 μM sodium vanadate and once with kinase buffer (20 mM Tris, pH 8.0, 10 mM MgCl₂, 1 mM DTT, 10 μM ATP). The immune complexes were resuspended in 60 μl of kinase buffer containing 10μCi [γ³²P] ATP (3,000 Ci/mmol; Amersham Corp.) and 15 μg of myelin basic protein (kindly provided by Michael Weber at the University of Virginia, Charlottesville, VA). Reactions were incubated at room temperature for 20 min and were terminated by the addition of gel loading buffer (4% SDS, 80 mM DTT, 10% glycerol).

Histone H1 Kinase Assays

Crude MPF extracts were prepared by homogenizing 5–20 oocytes with 10–40 μl of extraction buffer (80 mM β-glycerophosphate, 20 mM EGTA, 15 mM MgCl₂, 20 mM Hepes, pH 7.2, 1 mM ATP, 1 mM DTT, 5 mM NaF) and centrifuging at 16,000 g for 5 min at 4°C. 2 μl of extract was transferred to 50 μl of stabilization buffer (80 mM β-glycerophosphate, 20 mM EGTA, 15 mM MgCl₂, 20 mM Hepes, pH 7.2, 1 mM DTT, 2.5 mM PMSF, 20 μM leupeptin, 10 μM protein kinase A inhibitor). The histone H1 kinase assay was performed by adding 10 μl of stabilized extract to 6 μl of a mixture containing 2 μg histone H1 (Sigma Immunochemicals), 1 mM ATP, and 1.5 μCi of [γ³²P] ATP. The reaction was incubated at room temperature for 15 min and then terminated with sample buffer. Samples were resolved by SDS-PAGE (10% polyacrylamide gel) and phosphorylated histone H1 was detected by autoradiography.

Results

Induction of a Signal Pathway Involving Xenopus Raf during Oocyte Maturation

Previous studies have shown that *Xenopus* Raf mRNA is maternally expressed and abundant in oocytes (39). It has also been shown that *Xenopus* MAPK, Xp42, becomes activated during progesterone-induced oocyte maturation (23, 27, 54, 55). Therefore, we initiated experiments to investigate the functional relationship between Raf and MAPK during *Xenopus* oocyte maturation. Oocytes were stimulated with progesterone and collected at 1-h intervals over a 5-h time period. The stimulated oocytes were then lysed and examined by Western blot analysis using antibodies specific for Raf, MAPK or phosphotyrosine. As shown in Fig. 1, the *Xenopus* Raf protein is present in unstimulated oocytes and undergoes a shift in electrophoretic mobility at 3 h after progesterone treatment (∿0.5–1.0 h before GVBD₅₀). Furthermore, the shift in *Xenopus* Raf mobility was found to be coincident with tyrosine phosphorylation of MAPK and a shift in its electrophoretic mobility (Fig. 1). In mammalian cells, the shift in the electrophoretic mobility of Raf-1 has been found to be the result of hyperphosphorylation and
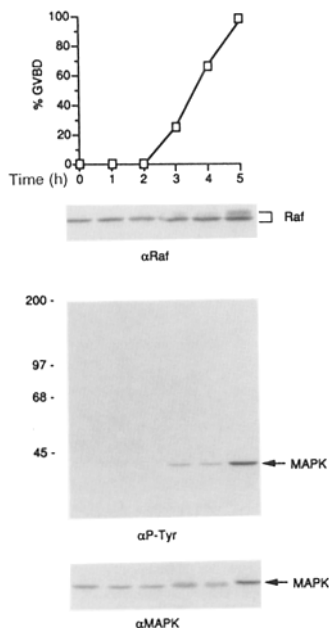
correlates with an increase in Raf kinase activity (47). Likewise, the shift in MAPK mobility and phosphorylation of MAPK on tyrosine residues is concurrent with MAPK activation (6, 12, 52, 55).

Oncogenic Raf-1 Can Induce Meiotic Maturation of Xenopus Oocytes

Since *Xenopus* Raf appears to become activated during oocyte maturation, we next asked whether introduction of a constitutively active oncogenic form of the human Raf-1 protein could induce maturation of oocytes in the absence of progesterone. Deletion of 305 amino acids from the amino-terminal regulatory region of Raf-1 has been shown to gener-
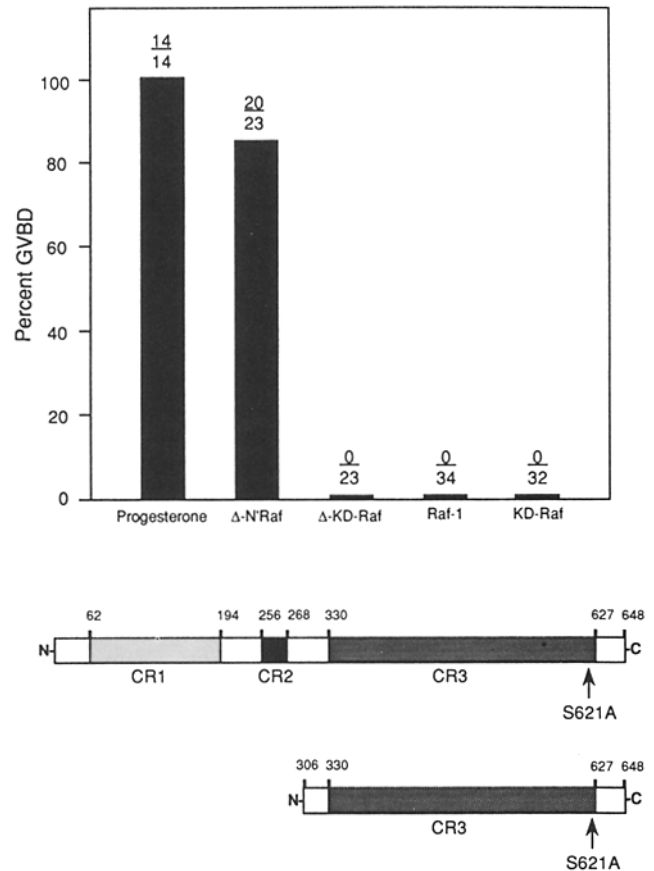

Figure 2. Induction of oocyte maturation by oncogenic Raf-1 RNA. Fully grown oocytes were either stimulated with progesterone (2 μg/ml) or microinjected with 30 ng of capped transcripts encoding the following proteins: oncogenic truncated Raf-1 (Δ-*N'Raf*), a kinase-defective form of oncogenic Raf-1 (Δ-*KD-Raf*), full length human Raf-1 (*Raf-1*), or a kinase-defective full length Raf-1 (*KD-Raf*). The percentage of oocytes undergoing GVBD are represented by the histogram bars, and the ratio of the number of oocytes with GVBD to the number injected is displayed above each bar. The full length Raf-1 peptide and the truncated version of Raf-1 (Δ-*N'Raf*) are depicted schematically below the histogram. The amino (*N*) and carboxyl (*C*) termini are labeled as well as the three evolutionarily conserved regions (*CR1, CR2,* and *CR3*) found in all Raf proteins (for review see Morrison [45]). The location of the serine to alanine substitution at position 621 (*S621A*) is denoted by an arrow for both the truncated and full length kinase-defective mutants (Δ-*KD-Raf* and *KD-Raf*, respectively).

Figure 1. Progesterone-induced GVBD is coincident with *Xenopus* Raf and MAPK, Xp42, activation. 80 oocytes were treated with progesterone (2 μg/ml) and the percentage of oocytes undergoing GVBD was determined as a function of time. At the indicated time, five oocytes were arbitrarily collected and lysed. Samples were resolved by SDS-PAGE (7.5% polyacrylamide gel), using 2.0 oocyte equivalents of lysates per lane, and examined by Western blot analysis using specific antibodies for Raf (α-Raf), MAPK (α-MAPK) or phosphotyrosine (αP-Tyr). Molecular weight markers are indicated at the left side of the αP-Tyr autoradiograph.

ate a constitutively active kinase capable of transforming mammalian fibroblasts at a high frequency (29, 68, 69). We injected fully grown oocytes with 30 ng of RNA encoding either full length or truncated versions of the human Raf-1 protein (Fig. 2, *Raf-1* and Δ-N'Raf, respectively). No GVBD was observed in oocytes injected with the full length Raf-1 RNA, while >80% of the oocytes injected with the truncated Δ-N'Raf RNA underwent GVBD within 12 h (Fig. 2).

It was important to confirm that the ability of the Δ-N'Raf RNA to induce oocyte maturation was due to the constitutive kinase activity of the truncated Raf-1 protein, Δ-N'Raf. We therefore injected oocytes with RNA encoding kinase-defective versions of the full length and truncated human Raf-1 proteins; designated KD-Raf and Δ-KD-Raf, respectively. Both the KD-Raf and Δ-KD-Raf mutants have a serine to alanine substitution at position 621 in the Raf-1 catalytic, CR3, domain. Serine 621 is a critical in vivo site of phosphorylation in all Raf proteins, and mutation of this residue inactivates the serine/threonine kinase activity of Raf-1 (47a). In addition, introduction of the S621A mutation in the Δ-N'Raf protein has been found to inactivate the serine-threonine kinase activity of this protein such that it is no longer able to transform mammalian fibroblasts (Morrison, D., and J. Fabian, unpublished data). As expected, oocytes injected with RNA encoding either KD-Raf or Δ-KD-Raf did not undergo GVBD.

Kinase-defective Raf-1 Inhibits Activation of MAPK during Maturation

When expressed in NIH-3T3 cells, an enzymatically inactive human Raf-1 protein has been shown to act in a dominant negative manner, inhibiting growth factor induced proliferation and transformation mediated by oncogenic ras proteins (34). Similarly, we used the kinase-defective mutant, KD-Raf, described in the above section to determine whether Raf function is required for *Xenopus* MAPK, Xp42, activation during oocyte maturation.

Stage VI oocytes were either left uninjected or microinjected with 30 ng of capped RNA encoding KD-Raf and maintained overnight. Oocytes injected in this manner expressed the KD-Raf protein at levels 15–30-fold higher than the endogenous *Xenopus* Raf protein (data not shown). These oocytes were either treated with progesterone or injected with RNA encoding an oncogenic truncated form of the hepatocyte growth factor receptor (Tpr–Met), Ha-Ras^V12, or Δ-N'Raf. In oocytes stimulated by progesterone, Tpr-met, and Ha-ras^V12, KD-Raf reduced detectable MAPK tyrosine phosphorylation by 95–100% relative to oocytes lacking KD-Raf and prevented the electrophoretic mobility shift of MAPK (Fig.3). Moreover, phosphorylation of myelin basic protein by MAPK immune complexes was markedly reduced in oocytes injected with KD-Raf (Fig. 3). Inhibition of MAPK activation was also observed in oocytes expressing KD-Raf along with EGFr in the presence of ligand (data not shown). Activation of MAPK by ΔN'Raf was not blocked by KD-Raf (Fig. 3). Furthermore, injection of the Δ-N'Raf RNA induced a shift in electrophoretic mobility of the Δ-N'Raf protein as well as that of the KD-Raf product. This result demonstrated that the constitutively active Raf is not inhibited by the presence of KD-Raf.

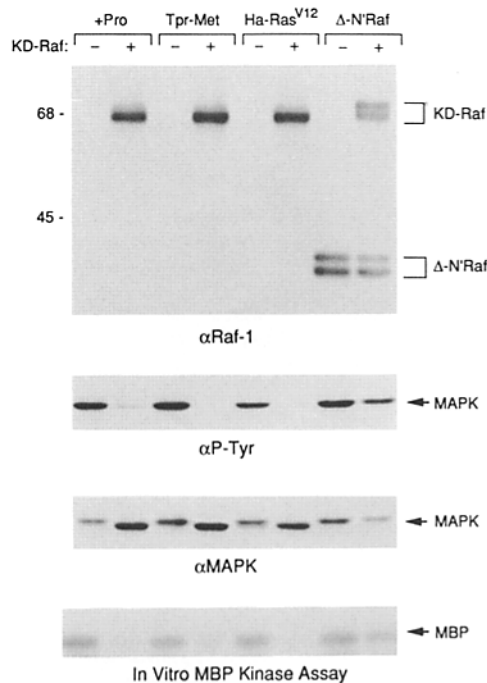Collectively, these findings indicate that the KD-Raf pro-

Figure 3. Inhibition of MAPK activation by kinase-defective Raf-1 (*KD-Raf*). Oocytes preinjected with KD-Raf (+), or not (−), were either treated with progesterone or microinjected with the capped transcripts (30 ng/oocyte) encoding Tpr-Met, Ha-ras^V12, or Δ-N'Raf. After 12 h, oocytes were collected and lysates were prepared. Samples were resolved by SDS-PAGE (8.0% polyacrylamide gel), using 2.5 oocyte equivalents of lysate per lane, and examined by Western blot analysis using specific antibodies for Raf (α-Raf), MAPK (α-MAPK) or phosphotyrosine (α-P-Tyr). Molecular weight markers are indicated at the left side of the a-Raf autoradiograph. For the in vitro MBP kinase assay, lysates were prepared (10 oocytes per treatment) and MAPK proteins were immunoprecipitated with a MAPK specific antibody. The immunoprecipitates were then washed and an in vitro kinase assay was performed using myelin basic protein as a substrate. Samples were resolved by SDS-PAGE (12% polyacrylamide gel) and phosphorylated MBP was detected by autoradiography.

tein acts as a dominant negative inhibitor of MAPK activation and this supports a model whereby MAPK functions downstream of Raf during the meiotic maturation of *Xenopus* oocytes.

Kinase-defective Raf-1 Blocks GVBD Induced by the Activated Receptor Tyrosine Kinase Pathway But Not by Progesterone

Next, we determined whether Raf-1 function was essential for oocyte maturation. When oocytes previously microinjected with KD-Raf were stimulated with progesterone, GVBD occurred in 86% of the oocytes (Fig. 4), even though Raf-1 and MAPK function were blocked (Fig. 3). The histone H1 kinase activity associated with maturation promoting factor activity was also present (Fig. 4). The oocytes expressing only Ha-ras^V12, Tpr-met, or EGFr in the presence of EGF displayed GVBD and Histone H1 kinase activity, while those oocytes co-expressing KD-Raf did not (Fig. 4).
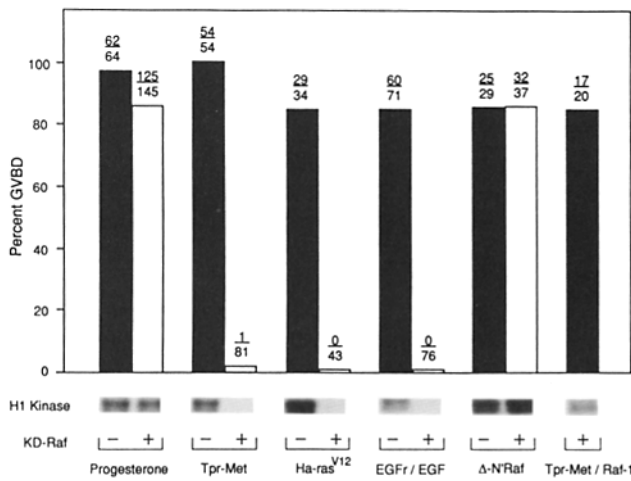
Figure 4. Inhibition of tyrosine kinases and other inducers of GVBD by kinase defective Raf-1 (KD-Raf). Oocytes preinjected with KD-Raf (+), or not (−), were either treated with progesterone or microinjected with the capped transcripts (30 ng per oocyte) encoding Tpr–Met, Ha-ras^{V12}, or Δ-N'Raf. In the case of the EGFr, EGFr RNA was injected 24 h before KD-Raf RNA injection (+), or not (−), then stimulated with EGF (0.1 μg/ml). GVBD was examined 12–18 h later. The ratio of the number of oocytes with GVBD to the total number injected is displayed above each bar. Histone H1 kinase assays were performed on extracts from five appropriately injected oocytes and the autoradiograph is displayed below each bar.

Figure 5. Effect of kinase-defective Raf-1 (KD-Raf) on progesterone-induced GVBD. 60 oocytes preinjected with either buffer (■) or 30 ng of KD-Raf RNA (□) were treated with progesterone (2 μg/ml) and GVBD was determined over a 7.5-h period. At the indicated times, oocytes were collected (5 oocytes per treatment) and lysates were prepared. Samples were resolved by SDS-PAGE (8.0% polyacrylamide gel), using 2.5 oocyte equivalents per lane, and examined by Western blot analysis using specific antibodies for Raf (α-Raf), MAPK (α-MAPK), or phosphotyrosine (αP-Tyr).

The differences in the ability to induce maturation was not due to variations in the levels of KD-Raf between progesterone stimulated or injected oocytes, since roughly equivalent levels of protein were present in lysates when assayed by Western blotting analysis (Fig. 3). Furthermore, inhibition of Tpr–Met-induced GVBD by KD-Raf did not appear to be due to a non-specific effect on translation caused by the KD-Raf RNA or protein for the following reasons: (a) KD-Raf did not inhibit translation of the Δ-N'Raf protein (Fig. 3); (b) GVBD was observed in oocytes injected with the KD-Raf RNA and subsequently injected with wild-type Raf-1 RNA and Tpr–Met (Fig. 4). Therefore, injection of KD-Raf appears to specifically inhibit maturation induced by activation of the RTK pathway, but not by the progesterone pathway in Xenopus oocytes.

Dominant Negative Raf Delays Progesterone Induced GVBD, but Maturation Progresses Through Meiosis II

To further examine the effect of KD-Raf on progesterone induced maturation, oocytes were preinjected with either KD-Raf RNA or buffer and were analyzed at 1.5-h intervals over a 7.5-h time period after progesterone treatment. Although 80–90% of the progesterone-treated oocytes expressing KD-Raf displayed GVBD, the GVBD$_{50}$ occurred 1.6–2.0 h later than buffer injected oocytes (Fig. 5). By Western blot analysis there was no detectable tyrosine phosphorylation or electrophoretic mobility shift in MAPK during oocyte maturation in the KD-Raf injected oocytes, while the normal phosphorylation and mobility shift was seen in the control
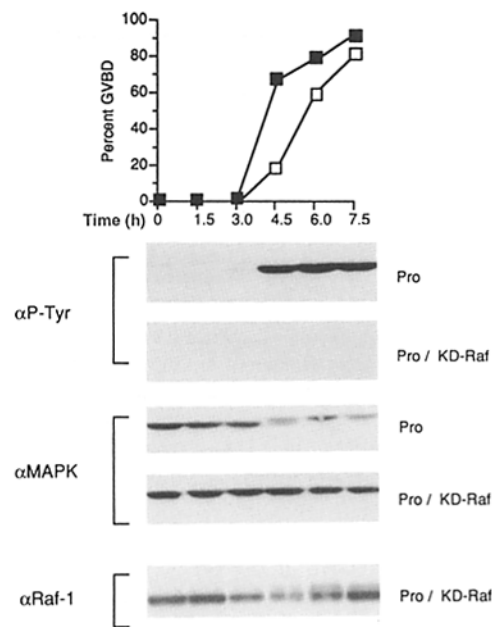
oocytes (Fig. 5). This result suggests that MAPK is not transiently activated during progesterone-induced maturation of KD-Raf injected oocytes. It is worth noting that a shift in the mobility of the KD-Raf protein occurred around the onset of GVBD (Fig. 5). The shift in mobility of the KD-Raf protein suggests that an upstream activator (or activators) of Raf is probably functioning but is unable to transmit its signal to the endogenous Xenopus Raf due to the presence of the KD-Raf protein.

Since GVBD occurs during meiosis I, we determined whether oocytes injected with KD-Raf RNA advanced through Meiosis I to metaphase of meiosis II. Extracts were prepared from progesterone-treated oocytes preinjected with either KD-Raf RNA or buffer and were assayed for histone H1 kinase and MPF activity over a 2.5-h period after GVBD. Regardless of whether the oocytes were injected with KD-Raf RNA, Histone H1 kinase activity decreased shortly after GVBD and reappeared, corresponding to meiosis II (Fig. 6). Histone H1 kinase activity remained high 6 h after GVBD (data not shown). Similar results were observed when the extracts were tested for MPF activity by microinjection into cycloheximide-treated fully grown oocytes (data not shown). MPF can induce GVBD even in the presence of cycloheximide (13, 72). Cycling of histone HI kinase activity between Meiosis I and II required approximately 1 h longer in the KD-Raf injected oocytes when compared to the buffer injected controls (Fig. 6).

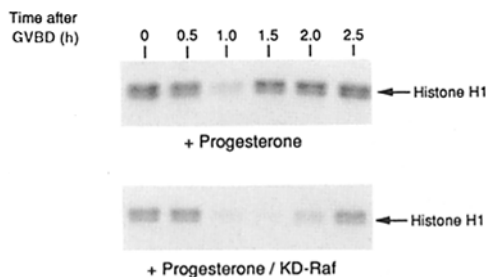The above results suggest that induction of meiotic matura-

Figure 6. Kinase-defective Raf (*KD-Raf*) does not block meiotic progression to metaphase II. Oocytes were injected with buffer or KD-Raf RNA (30 ng), and later stimulated with progesterone (2 µg/ml). At the indicated times following GVBD, groups of six oocytes were lysed and assayed for Histone H1 kinase activity. Samples were resolved by SDS-PAGE (10% polyacrylamide gel) and phosphorylated histone H1 was detected by autoradiography.

Figure 7. Mos is required for the activation of MAPK and the maximal electrophoretic mobility shift of Raf. Oocytes were either left uninjected (*U*) or injected with Mos-specific antisense (*A*) or sense oligonucleotides (*S*) and were cultured for 1.5 h. Oocytes were then injected with RNA encoding Tpr-Met, ΔN'Raf, or treated with progesterone (2 µg/ml). GVBD was determined and lysates were prepared 5–8 h later. Samples were resolved by SDS-PAGE (8.0% polyacrylamide gel), using 2.0 oocyte equivalents per lane, and examined by Western blot analysis using specific antibodies for Raf (α-Raf), MAPK (α-MAPK) or antiphosphotyrosine antibodies (αP-Tyr). The above results were reproducibly observed in three separate experiments.

tion by progesterone differs from induction by the tyrosine kinase-mediated pathway (Tpr-Met, EGFr, Ha-ras^{V12}) in that progesterone does not appear to require detectable Raf and MAPK function. Furthermore, Raf and MAPK activities do not appear to be necessary for cell cycle progression from G2/M of Meiosis I to M-phase of Meiosis II.

pp39^{mos} Synthesis Is Required for MAPK Activation and Affects the Shift of Xenopus Raf

pp39^{mos} can induce MAPK activation in cycloheximide treated oocytes (57) and its synthesis has been shown to be required for progesterone and tyrosine kinase-induced maturation (14, 33, 63). Therefore, we examined whether Mos is necessary for the electrophoretic mobility shift of Xenopus Raf and MAPK. Oocytes were microinjected with either Mos-specific antisense oligonucleotides or sense oligonucleotides and then either injected with Tpr–met RNA or treated with progesterone. Extracts were prepared 12 h later and subjected to Western blot analyses. Oocytes injected with antisense Mos oligonucleotides displayed 0–2% GVBD and did not show the maximal mobility shift of Raf when stimulated by progesterone or injection of the Tpr–Met RNA (Fig. 7). However, both progesterone and Tpr–Met did cause some intermediate shifted forms of Raf to appear (Fig. 7). As expected, GVBD (72–88%) and a maximal mobility shift of Raf was observed in the extracts from sense oligonucleotide injected oocytes stimulated by either reagent. Moreover, the tyrosine phosphorylation and electrophoretic shift associated with MAPK activity was also absent in the oocytes where Mos synthesis was precluded, despite the existence of some partially shifted forms of Raf induced by Tpr–Met and progesterone (Fig. 7). In contrast, oocytes pre-injected with Mos specific antisense oligonucleotides did undergo GVBD (91%) and possessed tyrosine phosphorylation of MAPK when injected with Δ-N'Raf RNA (Fig. 7).

Discussion

In this study we have examined the requirement for Raf and MAPK function during the maturation of Xenopus oocytes. Consistent with previous studies (4, 23, 27, 55), we found that in progesterone-treated oocytes, MAPK underwent sev-
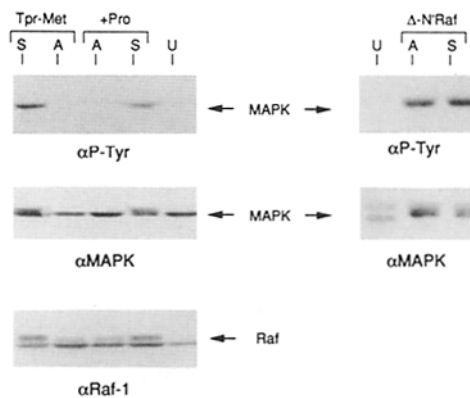
eral readily observable physical changes at the onset of GVBD. These changes included the tyrosine phosphorylation of MAPK, an alteration in its electrophoretic mobility and an activation of its serine/threonine kinase activity. Concurrent with MAPK tyrosine phosphorylation and at a time closely associated with MPF activation (∼0.7 GVBD$_{50}$) we observed a shift in the electrophoretic mobility of Xenopus Raf. In addition, we found that introduction of a constitutively active form of Raf, Δ-N'Raf, resulted in MAPK activation and GVBD. In contrast, expression of a kinase defective mutant, KD-Raf, inhibited MAPK activation induced by progesterone or by proteins associated with tyrosine kinase signaling pathways (Tpr–met, EGFr, Ha-ras^{V12}). This result demonstrates that MAPK functions downstream of Raf during the maturation of Xenopus oocytes and is consistent with observations of Raf function in mammalian somatic cells as well as genetic evidence from studies of Raf function during Drosophila development (16, 18, 32, 38, 49, 67, 71).

Previous studies have shown that the IGF-1 and progesterone activate MPF through different signal transduction pathways. For example, p21^{ras}, GAP, PKC-ζ are necessary for IGF-1 and not progesterone mediated activation of MPF (5, 17, 19, 20, 35). In this study, we found that expression of KD-Raf in oocytes blocked MPF activation and the resulting GVBD induced by Tpr-Met, EGFr, and Ha-Ras^{V12}, but failed to prevent progesterone-stimulated GVBD and progression of maturation to meiosis II. Progesterone induction of GVBD and the progression to meiosis II was delayed in the presence of KD-Raf, indicating that Raf function enhances the progesterone signaling of cell cycle progression.

The oncogenic ΔN'Raf product was found to induce MAPK and MPF activation in the presence of KD-Raf. In addition, injection of Raf-1 RNA rescued Tpr–Met induced maturation in oocytes expressing KD-Raf, demonstrating

that the inhibitory effects of KD-Raf are specific. Our data suggests that Raf and MAPK are common intermediates for both tyrosine kinase and progesterone signaling pathways. However, while Raf is an essential signaling component of the tyrosine kinase pathway, its activities may only expedite cell cycle progression induced by progesterone.

The pp39^{mos} product is a required component of both the tyrosine kinase and progesterone signaling pathways (14, 63). When introduced into oocytes, pp39^{mos} is able to induce MPF activation during meiosis I, but not meiosis II in the absence of protein synthesis (75). Furthermore, Mos is required for MPF activation throughout maturation (14, 33, 63). It has recently been shown that injection of Mos into oocytes activates MAPK and that the Mos product can phosphorylate MAPKK in vitro (57). The present study shows that the absence of Mos only partially inhibited the Raf mobility shift induced by progesterone and Tpr–Met, while preventing detectable tyrosine phosphorylation of MAPK. pp39^{mos} may be necessary for the full activation of Raf during oocyte maturation. Perhaps, Mos is required for the maximal activation of Raf that is necessary for signaling the activation of MAPK through MAPKK and possible MAPKKK. Consistent with this idea, Williams et al. (73) have shown that maximal Raf activation requires both ras-dependent and -independent signals (73). It may also be possible that Mos directly, or indirectly, phosphorylates Raf on critical serine or threonine residues necessary for full activation. Therefore, in the absence of Mos, progesterone and Tpr–Met may induce the partial or inappropriate phosphorylation of Raf that may effect Raf activity levels or substrate specificity. The mechanism by which Mos affects Raf activity may not be elucidated until the true substrate for Raf has been determined. Regardless, it is clear that Mos is required for detectable MAPK activation in both the tyrosine kinase and progesterone signaling cascades.

In somatic cells, Raf and MAPK appear to function in a signal transduction pathway which is initiated by growth factor binding to RTKs at the cell surface and results in the activation of transcription factors involved in cell proliferation (6, 10, 40, 52, 58, 66). An analogous role for Raf and MAPK in the maturation of *Xenopus* oocytes is unlikely since gene transcription is not needed until later in development. Indeed, our results suggest that Raf and MAPK may not be necessary for the progesterone-induced release of oocytes from arrest at G2/M of prophase I as well as for the progression to Meiosis II. However, it may very well be that Raf and MAPK function are critical to other processes involved in the production of mature eggs or during later stages of *Xenopus* development when gene transcription is required.

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