Paxillin Regulates Steroid-triggered Meiotic Resumption in Oocytes by Enhancing an All-or-None Positive Feedback Kinase Loop*

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Female fertility relies on precise regulation of oocyte meiosis. Immature oocytes are arrested at prophase I of meiosis until just prior to ovulation, when gonadotropin-induced signals within the ovary trigger them to re-enter the meiotic cycle. Oocytes progress through meiosis until metaphase II, at which point meiotic progression is again arrested, and mature oocytes are competent for ovulation and fertilization (1, 2). *Xenopus laevis* oocytes have long served as a model for studying the molecular signals that regulate meiosis, and many of these pathways now appear to be conserved in mammalian oocyte maturation as well (3, 4). Although many steroids promote *Xenopus* oocyte maturation *in vitro*, androgens likely serve as the physiological trigger *in vivo*, signaling through classic androgen receptors in a transcription-independent (non-genomic) fashion (5, 6). Thus, *Xenopus* oocytes not only serve as a superb model for studying the general principles of meiosis, but also as one of the few well accepted, biologically relevant examples of non-genomic steroid-mediated signaling.

What transcription-independent signals are modulated by androgens during meiosis? Steroids appear to trigger maturation in a “release of inhibition” fashion whereby oocytes are held in meiotic arrest by constitutive G protein signals that stimulate adenylyl cyclase to elevate intracellular cAMP (7–9). By mechanisms still not understood, CAMP inhibits kinase signaling cascades that are critical for meiotic progression (10–12). During ovulation, gonadotropins stimulate ovarian androgen production in *Xenopus*, leading to activation of androgen receptors, attenuation of the constitutive G protein signaling (and possibly activation of phosphodiesterases), and a drop in intracellular CAMP. Once CAMP is reduced, the downstream kinases are activated, leading to germinal vesicle breakdown (2, 13).

Another fascinating feature of this steroid-activated kinase cascade is that it constitutes a powerful, all-or-none positive feedback loop that amplifies small, reversible early signals into large, irreversible late effects (14). At the top of the cascade is MOS, an oocyte-specific mitogen-activated protein kinase kinase (MEK) similar to Raf (Fig. 7). Interestingly, *Mos* mRNA is present in immature oocytes, but virtually none is translated into stable MOS protein. Steroidos trigger increased polyadenylation of *Mos* mRNA in a process that involves interactions of regulatory proteins EG2 and cytoplasmic polyadenylation element-binding protein with the 3′-untranslated region of *Mos* mRNA (15–20). Increased polyadenylation of *Mos* mRNA leads to increased MOS protein expression. MOS then activates MEK1 (21), which in turn activates extracellular signaling-regulated kinase 2 (ERK2, or p42), and finally cyclin-dependent

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4 The abbreviations used are: MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; HA, hemagglutinin; UTR, untranslated region; dsRNA, double-stranded RNA; MOPS, 4-morpholinepropanesulfonic acid; GST, glutathione S-transferase.
kinase 1. Activated ERK2 then further increases MOS protein expression and activity, thus markedly enhancing the signaling cascade (22–24). Although this unusual positive feedback loop has been documented for years (2, 25–27), little progress has been made in identifying factors that regulate its activity.

To address the control of MAPK signaling in oocytes, we focused on the adapter protein, Paxillin. Interestingly, in growth-factor-stimulated mammalian somatic cells, Paxillin interacts with and regulates the activity of several components of the MAPK signaling cascade, including Raf, MEK, and ERK (28, 29). Paxillin also modulates many other physiological pathways, including matrix organization, cytoskeletal regulation, and focal adhesion turnover (30). Notably, Paxillin is an ideal candidate for a regulator of intracellular signaling, because it contains several protein binding domains, including many N-terminal leucine-rich LD motifs and C-terminal double zinc finger LIM domains (31–33). Furthermore, multiple phosphorylation sites have been identified, and phosphorylation appears to regulate both localization and function of Paxillin (34, 35).

Because Paxillin has been implicated as a regulator of MAPK signaling, we wished to determine whether it might be mediating the unusual steroid-triggered kinase feedback loop in oocytes. We detected Paxillin expression in *Xenopus* oocytes, and, through knockdown experiments, showed that Paxillin is required for oocyte maturation. Signaling studies revealed that Paxillin functions downstream of *Mos* mRNA polyadenylation but upstream of MOS protein expression. However, Paxillin is also phosphorylated downstream of MOS in a MEK-dependent manner, and this phosphorylation is necessary for full Paxillin function. Thus, Paxillin is a critical regulator of meiosis and plays an important role in the positive effects of MEK/ERK signaling on MOS protein expression and activity.

**EXPERIMENTAL PROCEDURES**

**Antibodies**—Anti-Mos (sc-86) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA), anti-HA (PRB-101) from Covance (Princeton, NJ), anti-phospho-p44/42 MAPK (#9101) and anti-total p44/42 MAPK (#9102) from Cell Signaling Technologies (Beverly, MA). Polyclonal antibodies directed to XePaxillin amino acids 468–484 were generated by Biosynthesis Inc. (Lewisville, TX).

**Plasmid Construction**—cDNAs encoding XePaxillin, XeMOS, and the 3′UTR of *Mos* mRNA were cloned into pcDNA3.1(+) (Invitrogen) for eukaryotic expression and pGEM HE (from L. Jan, University of California, San Francisco, CA) for *Xenopus* oocyte expression. Primers used to clone XePaxillin and XeMOS included sequence to incorporate an N-terminal HA tag. Serine-mutated versions of Paxillin were cloned using site-directed mutagenesis to convert serine residues to alanine.

**Oocyte Preparation**—Oocytes were harvested from female *X. laevis* (NASCO) and treated as described (Lutz et al. (7)). Briefly, follicular cells were removed by incubation of ovaries in 0.8 mg/ml collagenase A (Roche Applied Science) in modified Barth’s solution without Ca²⁺ for 3–4 h. Oocytes were then washed and incubated overnight at 16 °C in modified Barth’s solution containing 1 mg/ml Ficoll, 1 mg/ml bovine serum albumin, 100 units/ml penicillin, and 0.1 mg/ml streptomycin.

Testosterone-induced maturation assays were performed on stage V/VI oocytes from each preparation to determine sensitivity to steroid. Maturation was scored as germinal vesicle breakdown, which was visualized as a white spot on the animal pole of the oocyte. Twenty oocytes were used for each data point in all experiments.

**RNA Synthesis and Injections**—The pGEM HE constructs were linearized with Nhel, except for the Mos 3′UTR reporter, which was linearized with XbaI. Capped cRNA was transcribed *in vitro* with T7 RNA polymerase according to the manufacturer’s protocol (Promega). RNA was suspended in injection buffer (10 mM HEPES, pH 7.4), and Stage V/VI oocytes were injected with 20 ng of cRNA using a Drummond automatic injector. For double-stranded RNA (dsRNA) injections, equal concentrations of forward and reverse XePaxillin cRNA were mixed, heated to 90 °C, and allowed to slowly cool to 30 °C. Oocytes were then injected with 40 ng of dsRNA. The sequences of the sense and antisense Paxillin oligonucleotides were C*T*A*GA-GGCGACATGGATGATCT*G*G*A and T*C*C*AGATCAT-CCATGTCCGCTT*C*T*Ag, respectively, with phosphorothioate bonds indicated by asterisks.

Oocytes were injected with 25 ng of oligonucleotides in each experiment. For the rescue studies, XePaxillin was recloned so that nucleotides at wobble positions in the first six codons were changed, preserving the amino acid sequence but preventing antisense oligonucleotides from binding. In each rescue experiment, oocytes were co-injected with 25 ng of oligonucleotide and 20 ng of rescue cRNA. After all injections, oocytes were incubated at least 36–48 h before beginning any assay.

**Steroid Maturation Assays**—Maturation assays were conducted by incubating 20 oocytes per condition with the indicated concentration of testosterone (Steraloids) or ethanol in modified Barth’s solution for 12–16 h, at which time oocytes were scored for germinal vesicle breakdown (visualization of a white spot on the animal pole). Dilutions were performed such that ethanol concentration was held at 0.1%. In experiments with the MEK inhibitor PD98059 (Calbiochem), oocytes were pretreated with 50 μM PD98059 or 0.1% Me₂SO for 1 h. Oocytes were then incubated with the indicated concentration of testosterone and either PD98059 or Me₂SO.

**Western Blots**—Oocytes were incubated with steroid or vehicle, permeabilized in 20 μl/oocyte lysis buffer (1% Triton X-100, 50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 2 mM EDTA, 2 mM NaF, 0.5 mM sodium vanadate, 100 μg/ml phenylmethylsulfonyl fluoride), and microcentrifuged at 14,000 × g for 10 min to remove yolk and other debris. The cleared supernatants were then mixed 1:1 with 2× SDS buffer. The equivalent of 0.5 oocyte was loaded in each lane for SDS-PAGE, transferred to Immobilon-P membranes (Millipore), blocked in 5% TBST-milk for 1 h, then incubated with primary antibody overnight at 4 °C (1:5000 for anti-HA, 1:5000 for pre-immune and immune sera, 1:1000 for α-MOS, 1:2000 for α-phospho-p42 ERK2). Membranes were then incubated with horseradish peroxidase-conjugated secondary antibody for 1 h, and signal detected by ECL Plus (Amersham Biosciences). Total ERK blots were generated by stripping phospho-ERK blots, then incubating with 1:1000 α-total p42 ERK2 antibody. For phosphatase assays,
owocytes were permeabilized in oocyte lysis buffer without sodium vanadate, then 30 μl was treated with 10 units of calf intestinal alkaline phosphatase (New England Biolabs) for 1 h at 37°C. Samples were then mixed with an equal volume of 2 X SDS buffer and immunoblotted.

**Mos Polyadenylation Assays and Northern Blots**—Polyadenylation of Mos RNA was determined as described (22). Briefly, 36 h after dsPax or AS injections, each oocyte was injected with 10 ng of cRNA encoding the terminal 280 nucleotides of the 3’ UTR of Mos mRNA, and oocytes were allowed to recover for 2 h. Oocytes were then stimulated with testosterone and permeabilized every 4 h. RNA was extracted using 1 ml of TRIzol (Invitrogen), and 20 μg of total RNA was run on a 2.2% agarose formaldehyde gel in 1 X MOPS buffer for ~10 h at 60 V. RNA was transferred to a Nytran SuPerCharge nylon membrane (Schleicher & Schuell) by downward transfer in 20 X SSC (Schleicher & Schuell) and UV cross-linked. Membranes were pre-hybridized in ExpressHyb (Clontech) at 68°C for 30 min, then probed with [32P]dCTP-labeled Mos 3’ UTR DNA (Rediprime II, Amersham Biosciences) for 1 h, washed 2 X in 2 X SSC, 0.05% SDS, 2 X in 0.1 X SSC, 0.1% SDS, then subjected to autoradiography.

To confirm knockdown of Paxillin mRNA by Northern analysis, 20 μg of total RNA from sense and antisense injected oocytes were treated as in the polyadenylation assay with the following changes. Samples were run on a 1% agarose formaldehyde gel for 3 h at 90 V. The membrane was probed with [32P]dCTP-labeled Paxillin DNA encoding nucleotides 585–858. The membrane was stripped by adding to boiling 0.1% SDS, allowed to cool for 15 min, and then repeated. The membrane was pre-hybridized again, then probed with a [32P]dCTP-labeled *Xenopus* glyceraldehyde-3-phosphate dehydrogenase DNA probe encoding nucleotides 171–471.

**Cell Culture and Transfection**—COS-7 cells (ATCC) were maintained at 37°C in Dulbecco’s modified Eagle’s medium (Fisher Scientific) containing 10% fetal bovine serum, 100 IU/ml penicillin, and 0.1 mg/ml streptomycin (Invitrogen). Transfections were performed in 6-well plates using Lipofectamine reagent (Invitrogen). Each well was transfected with 1 μg of total DNA as indicated. After overnight incubation in 10% serum, cells were serum-starved for 24 h. Cells were then treated with either 50 μM PD98059 or 0.1% Me2SO in serum-free Dulbecco’s modified Eagle’s medium for 2 h. Cells were washed 2 X with ice-cold phosphate-buffered saline (pH 7.4), and permeabilized in 300 μl of oocyte lysis buffer. Wells were scraped, cell debris was removed by centrifugation, and the cleared lysates were mixed 1:1 with 2X SDS buffer and immunoblotted as described.

**GST Proteins and in Vitro Kinase Assays**—XePaxillin was cloned into pGEX4T-1 (Amersham Biosciences) and transformed into BL21. Transformed bacteria were then induced with 100 μM isopropyl 1-thio-β-D-galactopyranoside for 2 h and permeabilized in phosphate-buffered saline including 10 mM dithiothreitol, 1 μM/ml leupeptin, 1 μM/ml aprotinin, 1 μM/ml pepstatin A, and 100 μg/ml phenylmethylsulfonyl fluoride using an Emmulsiflex C5. The cleared lysate was incubated with glutathione-Sepharose beads and protein eluted with 10 mM glutathione in 50 mM Tris, pH 8.0, 1 mM EDTA.

One microgram of GST protein was incubated in MAPK buffer with either 100 ng of inactive ERK (Cell Signaling Technologies) or active ERK (New England Biolabs) per the manufacturer’s protocol. 10 μCi of γ-ATP was added to each reaction, and the kinase reaction incubated at 30°C for 1 h followed by mixing with an equal volume of 2 X SDS buffer. Samples were run on 10% gels by SDS-PAGE, dried, then analyzed by autoradiography. To confirm equal loading, reactions were run in duplicate without radioactive ATP, and samples were analyzed by Western blot using 1:1000 anti-Paxillin antibody (Santa Cruz Biotechnologies).

**Immunohistochemistry**—Oocytes were injected with either vehicle, 40 ng of dsXePax RNA, or 25 ng of the indicated oligonucleotides. After 48 h, the oocytes were fixed in paraffin, sectioned, and mounted on slides (Molecular Pathology Core Facility, University of Texas Southwestern). After blocking with horse serum, slides were incubated overnight with a 1:500 dilution of rabbit serum containing an anti-XePaxillin antibody directed against residues 468–484 or its corresponding pre-immune serum from the same rabbit. XePaxillin was then detected using the Vectastain ABC kit per the manufacturer’s protocol and viewed and photographed using a Nikon microscope and digital camera.

**RESULTS**

**Paxillin Is Expressed in X. laevis Oocytes and Is Required For Steroid-triggered Maturation**—A cDNA encoding a 539-amino acid isoform of *X. laevis* Paxillin (XePaxillin) protein (BAA96456) was cloned from oocyte mRNA using reverse-transcription-PCR (36). XePaxillin shares ~80% overall identity with human Paxillin, with 100% identity in two SH2 binding sites, five LD motifs, and four LIM domains (Fig. 1). Northern blot analysis confirmed the presence of Paxillin mRNA, with two isoforms detected (Fig. 2E), and Paxillin mRNA expression was unaffected by steroid treatment (data not shown). Immunohistochemistry using antibodies against XePaxillin revealed specific staining throughout the oocyte, including both nuclear and cytoplasmic regions (Fig. 2C). Interestingly, the nuclear staining of XePaxillin was the strongest, consistent with some subcellular localization studies in mammalian somatic cells. Notably, the signal using the anti-XePaxillin serum was considerably stronger than that seen using the corresponding pre-immune serum (Fig. 2C), illustrating the specificity of the immunohistochemistry. Finally, the anti-Paxillin serum (but not the corresponding pre-immune serum) recognized the overexpressed C-terminal half of XePaxillin, by Western blot
To determine whether Paxillin was required for steroid-induced oocyte maturation, overexpression and depletion studies were performed. Overexpression of Paxillin by cRNA injection had no effect on testosterone-induced maturation as compared with mock injected control oocytes. In contrast, knock-down of endogenous Paxillin expression by injection of full-length double-stranded XePaxillin RNA (dsRNA) dramatically inhibited oocyte maturation in response to testosterone (Fig. 2A). Significant reduction in Paxillin expression was confirmed by immunohistochemistry (Fig. 2C, left). Injection of nonspecific dsRNAs did not affect maturation (data not shown), confirming the specificity of the XePaxillin dsRNA injections. Of note, RNA interference in *Xenopus* and mouse oocytes has been used by us and others with excellent success, and with an absence of nonspecific effects (6, 37–41).

The inhibitory effect of reducing Paxillin expression by dsRNA injection was confirmed by knocking down Paxillin expression using antisense phosphorothioated oligonucleotides directed against the start codon of the XePaxillin mRNA. This technique has been used extensively to reduce mRNA and protein expression in *X. laevis* oocytes (42–44). Injection of the XePaxillin antisense, but not sense, oligonucleotides markedly reduced XePaxillin mRNA expression by Northern blot but did not affect mRNA expression of glyceraldehyde-3-phosphate dehydrogenase mRNA (Fig. 2E). As with the dsRNA injections, injection of the XePaxillin antisense oligonucleotides reduced endogenous Paxillin expression by immunohistochemistry (Fig. 2C, right) and inhibited steroid-induced oocyte maturation when compared with oocytes injected with the respective sense oligonucleotides (Fig. 2B). Importantly, the inhibitory effect of the antisense oligonucleotide injections on maturation were rescued by overexpression of XePaxillin from injected cRNA that contained alternative DNA sequences at the region recognized by the antisense oligonucleotide (Fig. 2B). XePaxillin expression was confirmed by Western blot (inset to Fig. 2B). The ability of overexpressed XePaxillin to

(Fig. 2D), further confirming the specificity of the antibody. Unfortunately, although it specifically detected endogenous Paxillin by immunohistochemistry, our antibody was not sensitive enough to detect endogenous Paxillin protein by Western blot.

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**FIGURE 2.** Paxillin is required for steroid-induced oocyte maturation. A, oocytes were injected with HA-tagged XePaxillin cRNA (XePax) to increase XePaxillin expression, double-stranded XePaxillin cRNA (ds XePax) to decrease XePaxillin expression, or 10 mM Hepes (Mock). After 48 h, oocytes were incubated with the indicated concentrations of testosterone and maturation (germinal vesicle breakdown) was scored after 16 h. B, oocytes were injected with either sense or antisense oligonucleotides without and with HA-XePaxillin cRNA and then treated as in A. Rescued oocytes contained HA-XePaxillin as measured by Western blot using an anti-HA antibody (inset). Results of dsRNA and antisense knockdown studies are representative of at least five experiments each. C, immunohistochemistry was performed on paraffin-embedded sections of mock, XePaxillin dsRNA, XePaxillin sense oligonucleotide, or XePaxillin antisense oligonucleotide injected oocytes. Sections were incubated with equal concentrations of rabbit anti-Paxillin antisera or the corresponding preimmune serum. These are representative photos of multiple slides. D, the anti-Paxillin, but not the corresponding pre-immune, serum recognizes the C-terminal half of XePaxillin when overexpressed in oocytes. Oocytes were injected with cRNAs encoding the indicated proteins and Western blots performed after 48 h. E, XePaxillin mRNA is present in sense-injected oocytes but disappears in oocytes injected with XePaxillin antisense oligonucleotides, as detected by Northern blot (upper panel). Northern blots correspond to the mRNA prepared for Fig. 2D. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels were unchanged (lower panel).
Whether Paxillin was important for steroid-triggered MOS protein expression and activity, we first tested cyclin B, to trigger oocyte maturation (13) (see Fig. 7). To determine where in this pathway Paxillin functions, we first tested whether Paxillin was important for steroid-triggered MOS protein expression and downstream ERK2 phosphorylation. MOS accumulation and ERK2 phosphorylation were not affected when XePaxillin was overexpressed in oocytes (Fig. 3A). However, in oocytes injected with XePaxillin dsRNA, and therefore with decreased endogenous Paxillin, steroid-induced MOS protein expression and ERK2 activation were significantly reduced (Fig. 3A). These inhibitory effects were also seen in oocytes depleted of Paxillin by antisense, but not sense, oligonucleotide injection (Fig. 3B), confirming the specificity of the dsRNA results. Furthermore, insulin-induced ERK2 phosphorylation, which is mediated in a MOS-independent fashion, was unaffected by the loss of Paxillin by dsRNA injection, suggesting that Paxillin plays a specific role in steroid-induced activation of ERK2 by MOS (Fig. 3A, last two lanes).

Because Paxillin was necessary for normal MOS protein expression, we next determined whether Paxillin regulated the first step of MOS activation, Mos mRNA polyadenylation. The endogenous Mos mRNA is over 3000 bp long, making detection of changes in polyadenylation difficult. To overcome this problem, a reporter assay was used whereby oocytes were injected with a small cRNA that contains ~280 nucleotides of the 3′UTR of Mos mRNA (22). This region includes the required cis-regulatory elements that confer polyadenylation of Mos mRNA in response to steroid stimulation. Therefore, in oocytes, the injected small cRNA will be polyadenylated in response to steroid, and increases in its size can be detected by Northern blot. Overexpression of Paxillin had no effect on polyadenylation when compared with control oocytes, as seen by similar time-dependent shifts in mobility (Fig. 3C). Surprisingly, loss of Paxillin, either by dsRNA or antisense oligonucleotide injection, also had no effect on Mos mRNA polyadenylation relative to mock or sense-injected oocytes (Fig. 3, C and D). These results suggest that Paxillin must be functioning downstream of Mos mRNA polyadenylation but upstream of MOS protein expression.

To confirm that Paxillin functions upstream of MOS, we attempted to rescue ERK2 phosphorylation and oocyte maturation in Paxillin-depleted oocytes by overexpression of MOS protein. Oocytes injected with Mos cRNA lacking the 3′UTR expressed MOS protein independent of steroid, leading to spontaneous activation of ERK2 and oocyte maturation (Fig. 3,
and F, sense). As expected, Mos cRNA injection rescued the inhibitory effects in oocytes injected with Paxillin antisense oligonucleotides (Fig. 3, E and F, antisense), resulting in equivalent expression of MOS, activation of ERK2, and rates of spontaneous maturation, as compared with sense-injected oocytes.

**Paxillin Is Phosphorylated in a MEK-dependent Fashion**—Because mammalian Paxillin serves as a MAPK scaffold in somatic cells, and phosphorylation of Paxillin is important for this function (28, 45), we determined whether Paxillin was phosphorylated during steroid-induced oocyte maturation. Oocytes were injected with HA-tagged XePaxillin cRNA, stimulated with testosterone, and solubilized every 2 h for detection of HA-tagged Paxillin by Western blot. After 8 h of steroid stimulation, a slower migrating form of Paxillin was observed that became even more abundant at 12 h (Fig. 4A). This higher migrating band disappeared with alkaline phosphatase treatment, suggesting that the change in Paxillin mobility was due to phosphorylation (Fig. 4A). Interestingly, the time at which Paxillin phosphorylation was detected coincided with the timing of significant MOS protein accumulation and the onset of germinal vesicle breakdown (Fig. 4C), providing further evidence that Paxillin function and MOS expression are linked.

Several reports have shown that phosphorylation of mammalian Paxillin occurs primarily on the N-terminal half of the protein (34, 46). Accordingly, the N-terminal, but not C-terminal, half of XePaxillin was phosphorylated upon stimulation with testosterone (Fig. 4B). Previous studies suggested that Raf-mediated phosphorylation of Paxillin in epithelial cells may require ERK activation (45, 47, 48). Because MOS is a germ cell-specific homologue of Raf, we tested whether downstream MEK activity was required for testosterone-induced phosphorylation of Paxillin. Oocytes treated with the MEK inhibitor PD98059 showed significantly reduced testosterone-induced maturation (data not shown), as well as almost complete loss of MOS protein expression and markedly reduced ERK2 activation (Fig. 4D). Furthermore, PD98059 almost completely abrogated Paxillin phosphorylation. Similarly, overexpression of MOS protein in oocytes was sufficient to activate ERK2 and promote phosphorylation of Paxillin (Fig. 4E), and PD98059 significantly delayed and

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**FIGURE 4.** Paxillin is phosphorylated late during maturation in a MEK-dependent fashion. A, oocytes were stimulated with 1 μM testosterone (to ensure maximum signaling) 40 h after injection of oocytes with HA-Paxillin cRNA. Oocytes were permeabilized at the indicated times, and 30 μl of the sample was treated with calf intestinal alkaline phosphatase. Equal amounts of lysate were analyzed by SDS-PAGE followed by Western blot with and anti-HA antibody. The higher mobility bands represent phosphorylated Paxillin. B, oocytes were injected with either HA-tagged cRNA encoding full-length (FL) Paxillin, the N-terminal half of Paxillin (N-Paxillin, residues 1–304), or the C-terminal half of Paxillin (C-Paxillin, residues 291–539). 40 h post-injection, oocytes were stimulated with 1 μM testosterone, permeabilized at the indicated times, and immunoblotted with HA antibody. C, lysates from A were probed with MOS antibody. D and E, 40 h post-injection, HA-Paxillin-expressing oocytes were pretreated with either Me2SO or 50 μM PD98059 for 2 h before stimulation with 1 μM testosterone (D) or injection with Mos cRNA (E). Either Me2SO or PD98059 was maintained in the media throughout the experiment. Oocytes were permeabilized at the indicated times and lysates probed for HA, MOS, and phospho-p42 ERK2. F, COS-7 cells were transfected with a total of 1 μg of DNA that consisted of pcDNA3.1+ cDNAs encoding either HA-Paxillin (0.5 μg) or HA-MOS (0.5 μg), or both HA-Paxillin and HA-MOS (0.5 μg each). After 18 h, cells were starved for 24 h, and then treated with either 50 μM PD98059 or Me2SO vehicle for 2 h. Cells were permeabilized, and equal amounts of lysate were probed for HA and phospho-ERKs. Phospho-ERK blots were stripped and re-probed for total ERK. Each experiment was reproduced at least three times with essentially identical results. Oocytes in A–E were from the same batch and treated as indicated at the same time.
Reduced these effects. Importantly, in oocytes injected with exogenous Mos cRNA, MOS protein accumulated more slowly in the presence of the MEK inhibitor, likely due to inhibition of the ERK2-mediated positive feedback loop that enhances MOS protein expression and activity. However, because MOS is such a potent activator of MEK1, PD98059 could only delay, but not abolish, ERK2 activity, likely explaining why Paxillin phosphorylation was reduced, but not completely eliminated, in Mos cRNA-injected oocytes.

To test whether MOS could also promote MEK-dependent phosphorylation of Paxillin in somatic cells, COS cells were transfected with either HA-tagged MOS, HA-tagged Paxillin, or both. After overnight serum starvation, cells were treated for 2 h with or without the MEK inhibitor PD98059. As in Xenopus oocytes, MOS expression led to phosphorylation of Paxillin, but here we observed only a modest increase in constitutive ERK1/2 activation (Fig. 4F). Nonetheless, as in oocytes, MOS-induced phosphorylation of Paxillin required MEK, as PD98059 blocked activation of ERK2 and eliminated the mobility shift of Paxillin. Because 2 h with PD98059 was sufficient to eliminate virtually all detectable MOS-mediated phosphorylation of Paxillin in COS cells, persistent MOS-induced MEK activity must be needed to keep Paxillin in its phosphorylated form.

**Paxillin Phosphorylation of Serine Residues 107/111 Is Required For Its Function in the Oocyte**—Having demonstrated that Paxillin is phosphorylated during oocyte maturation in a MOS- and MEK-dependent fashion, we next determined whether this phosphorylation was important for normal Paxillin function. In mammalian somatic cells, phosphorylation of two conserved tyrosine residues appears to be important for Paxillin’s actions in focal adhesion signaling and cell migration in somatic cells (amino acids 31 and 99 in XePaxillin (Fig. 1)) (28, 45). In addition, activation of the Raf-MEK-ERK cascade leads to phosphorylation of two serine residues of mammalian Paxillin (residues 107 and 111 in XePaxillin (Fig. 1)) (47, 49).

To determine the importance of these residues in regulating Xenopus oocyte maturation, point mutations were introduced in which the conserved tyrosine and serine residues were changed to phenylalanine and alanine, respectively (Fig. 1). Paxillin protein products containing the Y31F, Y99F, or Y31/99F mutations were still phosphorylated in response to testosterone (Fig. 5F). Nonetheless, as in oocytes, MOS-induced phosphorylation of Paxillin required MEK, as PD98059 blocked activation of ERK2 and eliminated the mobility shift of Paxillin. Because 2 h with PD98059 was sufficient to eliminate virtually all detectable MOS-mediated phosphorylation of Paxillin in COS cells, persistent MOS-induced MEK activity must be needed to keep Paxillin in its phosphorylated form.

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**Paxillin Phosphorylation and Function Depends on Serine Residues 107 and 111**—To test whether MOS could also promote MEK-dependent phosphorylation of Paxillin in somatic cells, COS cells were transfected with either HA-tagged MOS, HA-tagged Paxillin, or both. After overnight serum starvation, cells were treated for 2 h with or without the MEK inhibitor PD98059. As in Xenopus oocytes, MOS expression led to phosphorylation of Paxillin, but here we observed only a modest increase in constitutive ERK1/2 activation (Fig. 4F). Nonetheless, as in oocytes, MOS-induced phosphorylation of Paxillin required MEK, as PD98059 blocked activation of ERK2 and eliminated the mobility shift of Paxillin. Because 2 h with PD98059 was sufficient to eliminate virtually all detectable MOS-mediated phosphorylation of Paxillin in COS cells, persistent MOS-induced MEK activity must be needed to keep Paxillin in its phosphorylated form.

**Paxillin Phosphorylation of Serine Residues 107/111 Is Required For Its Function in the Oocyte**—Having demonstrated that Paxillin is phosphorylated during oocyte maturation in a MOS- and MEK-dependent fashion, we next determined whether this phosphorylation was important for normal Paxillin function. In mammalian somatic cells, phosphorylation of two conserved tyrosine residues appears to be important for Paxillin’s actions in focal adhesion signaling and cell migration in somatic cells (amino acids 31 and 99 in XePaxillin (Fig. 1)) (28, 45). In addition, activation of the Raf-MEK-ERK cascade leads to phosphorylation of two serine residues of mammalian Paxillin (residues 107 and 111 in XePaxillin (Fig. 1)) (47, 49).

To determine the importance of these residues in regulating Xenopus oocyte maturation, point mutations were introduced in which the conserved tyrosine and serine residues were changed to phenylalanine and alanine, respectively (Fig. 1). Paxillin protein products containing the Y31F, Y99F, or Y31/99F mutations were still phosphorylated in response to testosterone (data not shown). In contrast, an S107A mutation resulted in moderately reduced phosphorylation (Fig. 5A), and an S111A mutation resulted in significant loss of phosphorylation. Elimination of both serine residues (S107A/S111A) resulted in nearly complete loss of any slower migrating species in response to testosterone (Fig. 5A).

If phosphorylation at these serine residues is important for XePaxillin function, then the S107A/S111A Paxillin protein should be expected to have little or no activity. Oocytes were injected with the Paxillin antisense oligonucleotides to reduce endogenous Paxillin expression, and rescue studies were performed by re-injecting with cRNAs encoding wild-type or S107A/S111A XePaxillin. Overexpressed S107A/S111A XePaxillin in oocytes with reduced endogenous XePaxillin could not rescue maturation when compared with WT-Paxillin (Fig. 5B). Furthermore, S107A/S111A Paxillin was unable to rescue either MOS expression or activation of ERK2 as compared with WT-Paxillin res-
Paxillin Regulates Kinases to Mediate Oocyte Maturation

**FIGURE 6.** p42 ERK phosphorylates Paxillin in vitro. A, GST fusion proteins of full-length wild-type Paxillin (WT-Pax), S107A/S111A Paxillin (S107/111A-Pax), or GST alone were incubated with either inactive or active ERK2 in the presence of 10 μCi of [32P]ATP. No phosphorylation of GST alone was observed (data not shown). B, Western blots show equal, if not greater, loading of S107A/S111A Paxillin compared with WT-Paxillin.

**FIGURE 7.** Proposed model describing Paxillin function during oocyte maturation. Testosterone stimulation of oocytes via the classic androgen receptor triggers a decrease in intracellular cAMP. This drop in cAMP leads to increased polyadenylation of Mos mRNA, resulting in a small increase in MOS protein expression. MOS then activates MEK1, which in turn activates ERK2. Activated ERK2, and possibly other intracellular kinases, then phosphorylate Paxillin, which acts to further enhance MOS protein expression by either increasing MOS protein translation, decreasing MOS degradation, or both. This powerful positive feedback loop eventually leads to activation of the maturation promoting factor (MPF, or cyclin-dependent kinase 1 (CDK1), and cyclin B), resulting in meiotic resumption.

In vitro kinase assays to determine whether ERK2 could phosphorylate Xenopus Paxillin. GST fusion proteins of the wild-type and S107A/S111A XePaxillin proteins were expressed in bacteria and purified using glutathione-Sepharose beads. Activated, but not inactive, ERK2-phosphorylated GST-Paxillin significantly better than GST-S107A/S111A Paxillin (Fig. 6A). Multiple bands were observed due to phosphorylation of degradation products with an intact N terminus. This difference in phosphorylation was not due to variability in substrate amounts, because Western analysis showed that the quantity of S107A/S111A XePaxillin was at least equal to that of WT-Paxillin (Fig. 6B). Thus, ERK2 is capable of phosphorylating either one or both of the serine residues 107 and 111 in vitro and may be doing so in vivo as well.

**DISCUSSION**

The use of multidomain scaffold proteins is a key mechanism by which cells translate multiple extracellular stimuli into a complex array of intracellular signaling events. Paxillin serves as an excellent example of how these scaffolding proteins function. Due to the high number of protein binding motifs interspersed throughout the protein, Paxillin can recruit a large number of signaling intermediates and effector proteins into a single complex to allow for efficient and timely activation of multiple pathways. In addition, phosphorylation of Paxillin regulates which molecules are recruited and the subcellular compartment in which the complex localizes (30). Thus, Paxillin integrates multiple upstream signals into downstream signals that regulate a myriad of processes, including cytoskeletal regulation, matrix organization, cell motility, and gene expression.

One of the most interesting regulatory functions attributed to Paxillin is its role in regulating MAPK signaling. Under many conditions, Paxillin is critical for efficient activation of the MAPK cascade. For example, Paxillin-deficient fibroblasts show reduced fibronectin-induced ERK activation (51). In addition, Paxillin serves as a regulated MAPK scaffold that binds all three components of the MAPK cascade, Raf, MEK1, and ERK, upon growth factor stimulation of epithelial cells (28, 45).

We show here that Paxillin also plays a crucial role in the MAPK signaling responsible for the all-or-none switch that regulates steroid-induced oocyte maturation or meiotic progression. Our results demonstrate that Paxillin is a novel and essential regulator of steroid-triggered oocyte maturation. Signaling studies suggest that Paxillin functions upstream of MOS protein expression, because it is important for the accumulation of MOS and activation of the downstream kinases MEK and ERK2 (Fig. 3). In contrast, Paxillin also seems to be regulated downstream of MOS activation, because MEK-dependent phosphorylation of Paxillin on two serine residues appears to be critical for Paxillin to maintain elevated MOS expression and promote maturation (Fig. 5). How is it possible for Paxillin to be functioning both upstream and downstream of MOS? We propose a model whereby Paxillin is a critical positive regulator of the MOS/MEK/ERK2 feedback loop (Fig. 7). Upon steroid stimulation, Mos mRNA is stabilized by polyadenylation, leading to low level translation of MOS protein. MOS then activates the MAPK cascade, resulting in phosphorylation of Paxillin by a kinase downstream of MEK. Paxillin phosphorylation then enhances its ability to mediate MOS expression, allowing for accumulation of MOS at levels required to fully activate the MAP kinases and trigger resumption of meiosis by maturation-promoting factor.

What kinase is responsible for serine phosphorylation of Paxillin? One possibility is that MOS directly phosphorylates XePaxillin. However, the specific MEK inhibitor PD98059 blocked Paxillin phosphorylation both in oocytes and COS cells, suggesting that the Paxillin kinase is located downstream of MOS. Further, PD98059 does not block Raf activity and thus presumably would not inhibit the homologue MOS (52).

Since the Paxillin kinase acts downstream of MEK, then one candidate is ERK2. Accordingly, we demonstrated that ERK2 is capable of phosphorylating Xenopus Paxillin in vitro (Fig. 6), which is consistent with other studies looking at in vitro phosphorylation of mammalian Paxillin isoforms by ERK2 (29, 45).

5 M. Rasar, D. B. DeFranco, and S. R. Hammes, unpublished results.
Although we have no direct evidence that ERK2 phosphorylates XePaxillin in steroid-triggered oocytes, the observations that in vitro ERK2-mediated phosphorylation requires serine residues 107 and 111, and that XePaxillin might be a scaffold for MOS/MEK/ERK signaling, that ERK2 phosphorylates these or equivalent serine residues in vivo, indicate that ERK2 phosphorylates or equivalent serine residues in vivo. For example, ERK phosphorylates Xenopus Paxillin on serines in the frog kidney epithelial cell line A6 (50). In addition, ERK2 promotes phosphorylation of murine Paxillin on serine residues 126 and 130, equivalent to residues 107 and 111 in XePaxillin (47).

If Paxillin is allowing ERK2 signaling to enhance MOS protein expression, then how is it doing so? One possibility is that Paxillin is inhibiting MOS protein degradation. Some studies have suggested that, without ERK signaling, MOS is generally unstable and rapidly degraded, possibly in a ubiquitin-mediated proteasome-dependent manner (53–55). Upon activation with steroid, MOS degradation might be inhibited by phosphorylation of the serine 3 residue in an ERK2-dependent fashion (26, 56). However, in our hands, the proteasome inhibitor MG132 had no effect on steroid-triggered maturation or MOS expression in oocytes with normal or reduced Paxillin expression (data not shown), suggesting that Paxillin was unlikely to be significantly affecting proteasome-mediated MOS degradation.

Instead, Paxillin may be enhancing MOS translation. Support for this hypothesis is found in other studies, where ERK2 seems to enhance MOS translation (22), and Paxillin appears to enhance translation of polyadenylated mRNAs at focal adhesions (57).

Finally, similarities between our data and those examining Paxillin effects on MAPK signaling in somatic cells suggest that XePaxillin might be a scaffold for MOS/MEK/ERK signaling in oocytes. To test this hypothesis, we have attempted to demonstrate binding of XePaxillin to either MOS, MEK, or ERK by co-immunoprecipitation in oocyte extracts; however, we have been unable to detect consistent interactions between these molecules (data not shown). These negative data could be due to a variety of reasons, including difficulties with co-immunoprecipitation in oocyte extracts due to high levels of lipid and yolk contaminants, or weak/transient interactions that are disrupted upon cell lysis. More detailed precipitation studies are currently underway to determine whether these components directly bind to each other.

In sum, we have described a novel biological role for Paxillin in the process of meiotic maturation. Further, we have shown that Paxillin functions as a critical regulator of the all-or-none response observed during oocyte maturation due to positive effects on MAPK signaling. Because this MAPK effect shares many similarities to Paxillin effects in somatic cells, elucidating the role Paxillin plays in the maturation pathway will not only be helpful in understanding meiosis, but will likely reveal a more global role for Paxillin mediated MAPK signaling in both somatic and germ cell biology.

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