Dual-specificity protein phosphatases are implicated in the direct down-regulation of mitogen-activated protein kinase (MAPK) activity in vivo. Accumulating evidence suggests that these phosphatases are components of negative feedback loops that restore MAPK activity to low levels after diverse physiological responses. Limited information exists, however, regarding their post-transcriptional regulation. We cloned two *Xenopus* homologs of the mammalian dual-specificity MAPK phosphatases MKP-1/CL100 and found that overexpression of XCL100 in G2-arrested oocytes delayed or prevented progesterone-induced meiotic maturation. Epitope-tagged XCL100 was phosphorylated on serine during G2 phase, and on serine and threonine in a p42 MAPK-dependent manner during M phase. Threonine phosphorylation mapped to a single residue, threonine 168. Phosphorylation of XCL100 had no measurable effect on its ability to dephosphorylate p42 MAPK. Similarly, mutation of threonine 168 to either valine or glutamate did not significantly alter the binding affinity of a catalytically inactive XCL100 protein for active p42 MAPK in vivo. XCL100 was a labile protein in G2-arrested and progesterone-stimulated oocytes; surprisingly, its degradation rate was increased more than twofold after exposure to hyperosmolar sorbitol. In sorbitol-treated oocytes expressing a conditionally active 

\[ \text{Raf-DD:ER} \]

chimera, activation of the p42 MAPK cascade led to phosphorylation of XCL100 and a pronounced decrease in the rate of its degradation. Our results provide mechanistic insight into the regulation of a dual-specificity MAPK phosphatase during meiotic maturation and the adaptation to cellular stress.

**INTRODUCTION**

Members of the mitogen-activated protein kinase (MAPK) family play fundamental roles in the cellular response to diverse extracellular stimuli, including mitogens, cytokines, and environmental stresses (reviewed by Waskiewicz and Cooper, 1995; Ferrell, 1996; Lewis et al., 1998; Davis, 2000; Pearson et al., 2001). Activation of p42 MAPK and p44 MAPK in quiescent fibroblasts, for example, contributes to the induction of cyclin D expression and entry into the cell cycle (Pagès et al., 1993; Sun et al., 1994; Brondello et al., 1995; Cheng et al., 1998). Constitutive activation of MAPK cascades can cause cell transformation in fibroblasts (Cowley et al., 1994; Mansour et al., 1994) and, in a context-dependent and MAPK-specific manner, either promote or prevent apoptosis (Xia et al., 1995; Meier and Evan, 1998; Bonni et al., 1999). Thus, from a cellular perspective, precise regulation of MAPK activity can literally signify the difference between life and death.

The meiotic maturation of *Xenopus* oocytes provides an elegant system for studying the biochemistry of p42 MAPK regulation. Activation of the p42 MAPK cascade is important at multiple points during maturation: for the initial activation of Cdc2 triggering the G2-M transition (Sagata et al., 1988; Kosako et al., 1994b, 1996; Gotoh et al., 1995; Palmer et al., 1998); for Cdc2 reactivation and the suppression of DNA replication during the transition from meiosis 1 to meiosis 2 (Furuno et al., 1994); and for the maintenance of
MAPK-dependent Stabilization of XCL100

Both phosphorylations are accomplished by dual-specificity phosphatases, are similar in sequence to the tyrosine phosphatases, and dephosphorylate both residues in vitro via a catalytic mechanism analogous to that of tyrosine phosphatases (Ishibashi et al., 1992). Both phosphorylations are accomplished by dual-specificity mitogen-activated protein (MAP) kinase kinases, known as MEKs, MAPKks, or MKks (Crews et al., 1992; Kosako et al., 1992; Wu et al., 1992). Dual phosphorylation is necessary for full activation of MAPKs, and dephosphorylation of either residue is sufficient for their inactivation (Anderson et al., 1990; Posada and Cooper, 1992; Robbins et al., 1993). Several families of MAPK-directed phosphatases have been identified that can selectively dephosphorylate MAPks on threonine, tyrosine, or both. Members of the latter group, termed dual-specificity phosphatases, are similar in sequence to the tyrosine phosphatases, and dephosphorylate both residues in vitro via a catalytic mechanism analogous to that of tyrosine phosphatases (Ishibashi et al., 1992; Zhou et al., 1994; Denu et al., 1996a,b).

The prototype for the dual-specificity family of MAPK phosphatases is the MAP kinase phosphate-1 (MKP-1) protein. Mouse MKP-1 and its human homolog, CL100, were originally identified as immediate-early genes that were transcriptionally induced by growth factors, oxidative stress, and heat shock (Charles et al., 1992; Keyse and Emslie, 1992). Both proteins localize to the nucleus in transfected COS-1 cells and exhibit broad substrate specificities for p42/44 MAPK, JNK/SAPK, and p38/HOG1 (reviewed by Keyse, 1998).

Since the initial identification of MKP-1/CL100, at least nine other distinct MKP family members have been cloned and characterized. The individual MKPs differ slightly with regard to their specificity for different MAPKs, inducibility by extracellular stimuli, and subcellular localization. Expression of most, if not all, of these phosphatases is induced by the same stimuli that induce activation of MAPKs, suggesting that MKPs may be involved in negative feedback loops regulating MAPK activity. In support of this model, Bronnello et al. (1997) demonstrated that specific activation of the p42/p44 MAPK cascade in CCL39 fibroblasts up-regulates expression of MKP-1 and MKP-2, both of which are absent from quiescent cells.

Despite the ever-increasing number of MKP family members identified to date, little is known about the posttranscriptional mechanisms by which cells regulate MKPs to ensure the appropriate inactivation of distinct MAPKs. We sought to understand the regulation of one particular MKP in our biochemical system of choice, Xenopus oocytes. To this end, we cloned two highly similar Xenopus MKP-1/CL100 homologs and examined the status of an epitope-tagged XCL100 protein in oocytes exposed to either the maturation-inducing hormone progesterone or the hyperosmolar stress sorbitol. Whereas progesterone-stimulated oocytes activate both their p42 MAPK and JNK pathways, sorbitol-treated oocytes robustly activate only the JNK pathway (Bagowski et al., 2001). Xenopus oocytes therefore provide an attractive system for studying the posttranscriptional regulation of a dual-specificity MAPK phosphatase during the meiotic cell cycle and the adaptive response to hyperosmotic shock.

MATERIALS AND METHODS

Recombinant Proteins, Antibodies, and Other Reagents

The cDNA for a human Raf-1-estrogen receptor chimera, denoted ΔRaf-DD:ER (Bosch et al., 1997), was a generous gift from Martin McMahon (University of California, San Francisco Cancer Center, San Francisco, CA). The ΔRaf-DD:ER cDNA was subcloned into the pSP64(polyA) vector (Promega, Madison, WI) for in vitro transcription by Wen Xiong (Stanford University, Stanford, CA). The glutathione-S-transferase fusion protein GST-c-Jun (1–79), denoted GST-c-Jun, was expressed in Escherichia coli and purified to homogeneity by glutathione-Sepharose chromatography by Christoph Bagowski (Stanford University) as described (Bagowski et al., 2001). Hexahistidine-tagged Xenopus p42 MAPK(K57R) was purified by nickel chelate chromatography (Ferrell and Bhatt, 1997) and phosphorylated with [γ-32P]ATP as described previously (Sobhaser and Ferrall, 1999). Biphosphorylated, active murine p42 MAPK and lambda protein phosphatase were obtained from New England Biolabs (Beverly, MA) at specific activities of 5000 and 300 U/μg, respectively.

p42 MAPK antiserum DC3 was raised against a carboxy-terminal 12-aminoc acid peptide from the Xenopus p42 MAPK sequence (Hsiao et al., 1994). MEK antiserum 662 was raised in our laboratory as previously described (Hsiao et al., 1994). The polyclonal p90 Rsk (C-21) antibody and the polyclonal estrogen receptor α (HC-20) antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The monoclonal c-Myc (9E10) antibody was obtained from Babco (Richmond, CA). Alkaline phosphatase-conjugated secondary antibodies were obtained from Sigma (St. Louis, MO).

Progesterone, α-sorbitol, and cycloheximide were obtained from Sigma. 125I-labeled protein A and [32P]orthophosphate were obtained from ICN Pharmaceuticals (Irvine, CA). The MEK inhibitor U0126 (Promega) was reconstituted in dimethyl sulfoxide at a concentration of 10 mM and used at a final concentration of 50 μM, the lowest concentration that completely and reproducibly prevented p42 MAPK activation in oocytes (our unpublished observations; Gross et al., 2000; Bagowski et al., 2001).

Cloning of XCL100 cDNAs

Total ovary first-strand cDNA (kindly provided by Polly Wong, Stanford University) was used as a template in pairwise polymerase chain reactions (PCRs) with the degenerate oligonucleotides S1 (5’TGCCTCCXXA[A/C/T]/C/A/C/T/TTTG(A/G)/GG-3’), A1 (5’CC/TGTGXXCTCA/A/G/A/G/A/C/T/CT/TTT/T/AA[A/C/G/T-3’), and A2 (5’CC/CTCCXXA[G/A/G/A/G/A/C/G/T/T-3’), where X represents any of the four standard DNA nucleotides. The resulting PCR products were subcloned directly into the TA Cloning vector pCRII (Invitrogen, Carlsbad, CA). To confirm their sequence similarity to mouse MKP-1 and human CL100, individual cDNA clones were sequenced on both strands by the dyeoxynucleotide chain-termination method (Sanger et al., 1977), by using reagents and protocols supplied by Amersham Biosciences (Piscataway, NJ). One cDNA clone (designated Xm10) showing high-sequence homology to MKP-1/CL100 was used as a probe in screening a Xenopus ovary cDNA library constructed in the Uni-ZAP XR vector (Stratagene, La Jolla, CA).
Approximately $6 \times 10^5$ recombinants from this library were screened with a $^{32}$P-labeled Xmal probe following standard protocols (Sambrook et al., 1989). After three rounds of screening, 12 positive clones were plaque purified, and the corresponding cDNA inserts were excised from the Uni-ZAP vector as pBluescript SK(−) phagemids. Partial DNA sequencing of all 12 positive clones separated them into two distinct groups. The longest cDNA from each group was sequenced completely on both strands by the dideoxynucleotide chain-termination method (Sanger et al., 1977). These two clones, 2145 base pairs and 1999 base pairs in length, represented distinct, full-length inserts were excised from the Uni-ZAP vector as pBluescript SK(−). Positive clones were plaque purified, and the corresponding cDNA positive clones were plaque purified, and the corresponding cDNA was generated by PCR with the primers MKPmycA1 (5'-CAGGTAGCCCTA-3') and S2 (5'-GCTCGAGGCAGCTTGATGTAGT-3') -CAGAAG- was generated by PCR with the primers MKPmycA1 (5'-CAGGTAGCCCTA-3') and S2 (5'-GCTCGAGGCAGCTTGATGTAGT-3') -CAGAAG- was generated by PCR with the primers MKPmycA1 (5'-CAGGTAGCCCTA-3') and S2 (5'-GCTCGAGGCAGCTTGATGTAGT-3'). The 10-residue c-Myc epitope EQKLISEEDL was introduced at the M.L. Sohaskey and J.E. Ferrell X. laevis females were primed by injection of 50 μg/ml progesterone. GVBD was scored by the appearance of a white spot at the animal pole of the oocyte.

### Oocyte Microinjections

For expression of Myc-tagged XCL100 proteins in stage VI oocytes, the appropriate pSP64myc-XCL100 DNA was first linearized with PvuII. Capped, polyadenylated mRNA was then transcribed in vitro from this template by using the mMESSAGE mMACHINE SP6 kit (Ambion, Austin, TX) according to the manufacturer’s instructions, purified by phenol/chloroform extraction and isopropanol precipitation, and resuspended in diethylpyrocarbonate-treated water at 1 mg/ml. In vitro-transcribed mRNA was microinjected into the cytoplasm of stage VI oocytes, and injected oocytes were incubated in OR2 buffer (82.5 mM NaCl, 2.5 mM KCl, 1 mM CaCl₂, 1 mM Na₂HPO₄, 5 mM HEPES, pH 7.5) to allow protein expression. Oocytes were collected, frozen on dry ice, and stored at −80°C.

### Oocyte Lysis

Oocytes were thawed rapidly and lysed by trituration in ice-cold extraction buffer (EB: 0.25 M sucrose, 0.1 M NaCl, 2.5 mM MgCl₂, 20 mM HEPES, pH 7.2) containing 10 mM EDTA, protease inhibitors (10 μg/ml leupeptin, 10 μg/ml pepstatin, 1 μM phenylmethylsulfonyl fluoride), and phosphatase inhibitors (50 mM β-glyceroxylate, 1 mM sodium orthovanadate, 2 μM microcystin), where appropriate. Samples were clarified by centrifugation for 5 min in a Beckman E microcentrifuge (Fullerton, CA) with a right angle rotor. Crude cytoplasm was removed and immediately added to 0.2 volumes of 6× Laemmli sample buffer for subsequent SDS-PAGE, or diluted 1:2 in EB/0.2% Triton X-100 plus inhibitors for immunoprecipitation.

### SDS-PAGE and Immunoblotting

Samples were separated on 10% SDS polyacrylamide gels (acrylamide/bisacrylamide, 100:1), and the proteins were transferred to an Immobilon P (Millipore, Bedford, MA) blotting membrane. The membrane was blocked with 3% nonfat milk in Tris-buffered saline (150 mM NaCl, 20 mM Tris, pH 7.6) and incubated with primary antibody at a dilution of 1:500 (p42 MAPK and MEK) or 1:1000 (c-Myc, p90 Rsk, and estrogen receptor) for 1–2 h at room temperature. Blots were washed five times with Tris-buffered saline/0.05% Tween 20 and probed with either 125I-labeled protein A for autoradiography, or an alkaline phosphatase-conjugated secondary antibody for detection by enhanced chemiluminescence (ECL) (Amer sham Biosciences). For reprobing, blots were stripped by incubation with 100 mM Tris-HCl, pH 7.4, 100 mM 2-mercaptoethanol, and 2% SDS at 70°C for 40 min.

### Histone H1 and GST-Jun Kinase Assays

To determine Cdc2 activity, we performed kinase assays with histone H1 as a substrate. Oocytes were lysed in ice-cold EB plus inhibitors (20 μl/oocyte) and clarified in a Beckman E microcentrifuge for 5 min. Three oocyte equivalents were removed from the cytoplasmic fraction, diluted 1:2 in immunoprecipitation buffer (IPB: 15 mM EGTA, 10 mM MgCl₂, 25 mM Tris, pH 8.0, 0.1% Triton X-100, 0.1% bovine serum albumin) plus protease and phosphatase inhibitors, mixed with 18 μl of p13 Suc1 agarose, and incubated at 4°C for 3 h with rocking. After three washes with ice-cold immunoprecipitation buffer plus inhibitors, H1 kinase buffer (0.4 mM ATP, 0.25 μCi/μl [γ-32P]ATP, 0.5 μg/ml histone H1, 30 mM 3-(N-morpholino)propanesulfonic acid, pH 7.2, 7.5 mM MgCl₂, 0.3 mM dithiothreitol) was added to the beads, yielding a final reaction volume of ~30 μl. The reactions were incubated at 30°C for 10 min and stopped by the addition of 0.2 volumes of 6× Laemmli sample buffer. Proteins were electrophoresed on 12.5% SDS polyacrylamide gels (acrylamide/bisacrylamide, 29:1), transferred to Immobilon P

### Subcloning and Epitope Tagging of XCL100

The 10-residue c-Myc epitope EQKLISEEDL was introduced at the carboxy terminus of XCL100 as follows. A 185-base pair fragment containing the carboxy-terminal 146 base pairs of the XCL100 coding region followed by the Myc epitope and an in-frame stop codon was generated by PCR with the primers MKPmycA1 (5'-GAGAGACTTGATCAAGGTCCCTGAGTTT-GCTCGAGGCAGCTTGATGTAGT-3') and S2 (5'-GAGAGACTTGATCAAGGTCCCTGAGTTT-GCTCGAGGCAGCTTGATGTAGT-3'). The PCR product was digested with XbaI and PstI to generate a fragment of ~160 base pairs. The XCL100 cDNA in the pBluescript SK(−) vector was digested with HindIII and XbaI to obtain a 1.08-kb HindIII-XbaI fragment that was ligated to the XbaI-PstI digested PCR product, yielding an ~1.24-kb HindIII-PstI fragment containing the entire XCL100 open reading frame (plus 89 base pairs of the 5'-untranslated region) upstream of the carboxy-terminal Myc epitope. The ~1.24-kb HindIII-PstI XCL100 fragment was then ligated into the HindIII-PstI digested pSP64(polyA) vector to yield pSP64myc-XCL100. The fidelity of PCR was established by DNA sequencing.

### Site-directed Mutagenesis of XCL100

Site-directed mutagenesis of the XCL100 cDNA was performed using the MORPH site-specific plasmid DNA mutagenesis kit (Eppendorf-5 Prime, Boulder, CO) following the manufacturer’s protocol at the mutagen oligonucleotides used were: C260S (5'-GGAGAGACTTGATCAAGGTCCCTGAGTTT-GCTCGAGGCAGCTTGATGTAGT-3'), used to mutate cysteine 260 to serine; T168E (5'-GAGAGACTTGATCAAGGTCCCTGAGTTT-GCTCGAGGCAGCTTGATGTAGT-3'), used to mutate threonine 168 to valine. The fidelity of all mutagenesis procedures was verified by DNA sequencing.

### Isolation of Xenopus Oocytes and Induction of Maturation

*X. laevis* females were primed by injection of 50–67 IU of pregnant mare serum gonadotropin (Calbiochem) 3–10 d before oocyte removal. Stage VI oocytes, isolated and maintained as previously described (Sohaskey and Ferrell, 1999), were induced to reenter meiosis I and undergo germinal vesicle breakdown (GVBD) by treatment with 5 μg/ml progesterone. GVBD was scored by the appearance of a white spot at the animal pole of the oocyte.
(Millipore) blotting membranes, and 32P-labeled histone H1 was visualized by autoradiography.

To determine JNK activity, GST-Jun kinase assays were performed as described previously (Bagowski et al., 2001) and quantified with a Molecular Dynamics (Sunnyvale, CA) PhosphorImager.

**XCL100 Immunoprecipitation and Immunocomplex Phosphatase Assays**

For the experiment described in Figure 5A, lysates were prepared immediately after collection of G2- and M-phase oocytes expressing XCL100. Ten oocyte equivalents were diluted 1:1 in EB containing 0.2% Triton X-100 and added to 15 μl of washed protein A-agarose prebound to c-Myc antibody 9E10. After rotation for 2 h at 4°C, immunoprecipitates were washed twice with EB lacking phosphatase inhibitors and once with phosphatase buffer (40 mM HEPES, pH 7.5, 2 mM dithiothreitol, 0.1 mg/ml bovine serum albumin). Ten microliters of 2× phosphatase buffer was added together with 10 μl of either buffer or 32P-labeled, bisphosphorylated His6-p42 MAPK(K57R) (~200 nM final). Reactions were incubated at 30°C for 30 min with occasional gentle mixing and stopped by the addition of 0.2 volumes of 6× Laemmli sample buffer. Proteins were resolved by electrophoresis on 10% SDS polyacrylamide gels (acrylamide: bisacrylamide, 100:1) and transferred to Immobilon P (Millipore) by electrophoresis on 10% SDS polyacrylamide gels (acrylamide:bisacrylamide, 100:1) and transferred to Immobilon P (Millipore) blotting membranes. The loss of radiolabel in p42 MAPK(K57R) over time was visualized by autoradiography, quantified with a Molecular Dynamics PhosphorImager, and normalized to the radiolabel remaining in a control sample containing 32P-labeled p42 MAPK(K57R) alone. Immunoprecipitated XCL100 proteins were analyzed by 9E10 immunoblotting.

**Phosphoamino Acid Analysis**

32P-labeled XCL100 proteins were excised from blotting membranes and subjected to two-dimensional phosphoamino acid analysis at pH 1.9 in the first dimension and pH 3.5 in the second dimension (Boyle et al., 1991; Kamps, 1991). Radiolabeled XCL100 proteins and their constituent phosphoamino acids were visualized by autoradiography.

**Data Quantification and Curve Fitting**

To quantify XCL100 degradation (Figures 6 and 7), ECL-based densitometry was done using the Kodak Image Station 440CF and 1D Image Analysis Software (Eastman Kodak Company, New Haven, CT). Appropriate controls were evaluated to ensure that all data analyses were performed within the linear range of the system.

To generate the curves shown in Figures 6B and 7, and to estimate half-lives for XCL100, we fit the experimental degradation data to the equation \( t = \frac{\ln 2}{\ln \left(\frac{C_{t}}{C_{0}}\right)} \) by using KaleidaGraph (Abelbeck Software, Reading, PA). \( t \) represents the half-life of XCL100, and \( \ln 2 / \tau \) is the apparent zero order rate constant for the degradation reaction.

**RESULTS**

**Cloning of Two Xenopus Homologs of MAPK Phosphatase MKP-1/CL100**

As the first step in studying the regulation of a dual-specificity p42 MAPK phosphatase during the meiotic cell cycle, we cloned the Xenopus homolog of the murine/human dual-specificity phosphatases MKP-1/CL100. Using degenerate oligonucleotides derived from two carboxy-terminal stretches invariably conserved among the MKP family members MKP-1, CL100, and PAC-1 (Rohani et al., 1993), we performed PCR and amplified a single fragment of the predicted size from a template of stage VI oocyte first-strand cDNA. Screening an ovary cDNA library with this partial fragment yielded two highly similar yet distinct groups of sequences (our unpublished observations). These sequences are, on average, 77% identical in their predicted protein sequence to MKP-1 and CL100, including 100% identity within the canonical phosphatase catalytic domain (VHC-QAGISRS). Moreover, the two sequences are 94% identical to each other, suggesting that they correspond to pseudoalloploid alleles, a common manifestation of the tetraploid ancestry of *X. laevis* (Jones et al., 1988). For this reason, we named these clones XCL100α and XCL100β. The longest XCL100α and XCL100β cDNAs isolated were 2145 and 1999 base pairs in length, respectively, and represent full-length XCL100 sequences. Both cDNAs encode proteins of 369 amino acids (apparent molecular mass ~40 kDa) and contain termination codons upstream of and in-frame with the initiation codon ATG. Each cDNA also possesses, within its 3′-untranslated region, three copies of the mRNA destabilization sequence AUUUA (Shaw and Kamen, 1986) (our unpublished observations). The presence of AUUUA sequences is consistent with the finding that XCL100 mRNA levels decrease dramatically coincident with the first cleavage division in the fertilized egg (Lewis et al., 1995). We chose to concentrate our studies on XCL100α, which corresponds to the XCL100 sequence isolated by Lewis et al. (1995).

Coupled transcription/translation of this XCL100 cDNA in a rabbit reticulocyte lysate yielded one prominent band of ~40 kDa (our unpublished observations). In addition, a GST-XCL100 fusion protein selectively dephosphorylated *Xenopus* p42 MAPK on both tyrosine and threonine residues in a time-dependent manner in vitro (Wang et al., 1997; our unpublished observations). Phosphatase activity was prevented by the inclusion of the tyrosine phosphatase inhibitor sodium orthovanadate and was absent in a GST-CL100(C260S) fusion protein having its essential catalytic cysteine residue mutated to serine. These data support the identity of XCL100 as a dual-specificity p42 MAPK-directed phosphatase.

The expression levels of the endogenous XCL100 protein are relatively low in G2- and M-phase oocytes (Lewis et al., 1995). Therefore, we have used an epitope-tagged XCL100 protein as a surrogate for the endogenous phosphatase in our experiments.

**Overexpression of XCL100 Prevents Progesterone-induced Oocyte Maturation**

Having established its activity as a p42 MAPK phosphatase in vitro, we next asked whether XCL100 could function as a p42 MAPK-inactivating protein in vivo. To do this, we exploited the ability of fully grown *Xenopus* oocytes to undergo maturation in response to progesterone treatment. Several groups have reported a requirement for activation of the p42 MAPK cascade in progesterone-induced maturation (Sagata et al., 1988; Kosako et al., 1994b; Gotoh et al., 1995). We reasoned that if p42 MAPK activity were indeed important for this process then overexpression of XCL100 in oocytes should prevent p42 MAPK activation and inhibit meiotic progression.

We microinjected in vitro-transcribed mRNA encoding a Myc-tagged version of either wild-type or catalytically inactive (C260S) XCL100 protein into immature stage VI oocytes,
shown in Figure 1A, overexpression of the p42 MAPK substrate p90 Rsk, and Cdc2/cyclin B. As several key M-phase protein kinases, including p42 MAPK, tored biochemically by examining the activation state of animal pole of the oocyte. Meiotic progression was monitored visually by GVBD, the appearance of a white spot at the promtation, we consistently observed GVBD in a subpopulation of oocytes injected with either water alone or XCL100(C260S) mRNA matured with normal kinetics. Interestingly, activation of MEK, the relevant mitogen-activated protein kinase kinase in CL100-injected oocytes, was also blocked in XCL100-injected oocytes (our unpublished observations). This latter finding further supports the existence of a positive feedback loop operating within the p42 MAPK cascade (Gotoh et al., 1995; Matten et al., 1996; Roy et al., 1996; Howard et al., 1999).

Immunoprecipitation of the XCL100 and XCL100(C260S) proteins from G2- and M-phase oocytes demonstrated that XCL100(C260S) stably binds only the bisphosphorylated, active form of p42 MAPK (Figure 1B). XCL100(C260S) thus acts as a “substrate trap” (Sun et al., 1993), forming a stable complex with its putative substrate, active p42 MAPK. The expression levels of XCL100 protein were markedly lower in M-phase oocytes than in G2-phase oocytes (Figure 1B), suggesting that the phosphatase may be degraded in a progesterone-dependent manner (see below). Significantly, neither MEK, nor p90 Rsk, nor Cdc2/cyclin B were dephosphorylated by XCL100 in vitro (Huang, Sohaskey, and Ferrell, unpublished observations) or in Xenopus oocyte extracts (Alessi et al., 1993). Together, these results suggest that overexpressed XCL100 blocks progesterone-stimulated meiotic maturation by directly dephosphorylating and inactivating p42 MAPK or a p42 MAPK-related protein, such as JNK.

Although overexpressed XCL100 strongly inhibits maturation, we consistently observed GVBD in a subpopulation (typically 10–20%) of oocytes injected with XCL100 mRNA (Figure 1A, bottom). Moreover, none of these GVBD-positive oocytes contained detectable levels of active p42 MAPK as measured by immunoblot analysis (our unpublished observations). These data support three other groups’ findings that p42 MAPK activation, despite its proposed role in facilitating an unperturbed meiotic cell cycle, may be dispensable for progression into meiosis 1 in some oocytes (Fabian et al., 1993; Fisher et al., 1999; Gross et al., 2000).

Cell Cycle-dependent Phosphorylation of XCL100

Another intriguing observation to arise from these results concerns the reduced mobility of the XCL100(C260S) protein in mature oocytes (Figures 1B and 2A). Because phosphorylation is a frequently exploited mechanism for signal transduction in eukaryotic cells (Hunter, 1995), we investigated whether the progesterone-dependent mobility shift of XCL100(C260S) was attributable to phosphorylation. Lysates from mature oocytes expressing XCL100(C260S) were treated with lambda protein phosphatase (APP), a nonspecific protein phosphatase, and analyzed by SDS-PAGE and immunoblotting. Treatment with APP caused the more...
slowly migrating forms of XCL100(C260S) to collapse down into a single band, an effect that was prevented by the inclusion of millimolar concentrations of the phosphatase inhibitors sodium orthovanadate and sodium fluoride (Figure 2B). Surprisingly, the single band resulting from AIP treatment migrated more rapidly than that seen in untreated G2 lysates, suggesting that XCL100(C260S) may also be phosphorylated in G2-arrested oocytes.

To test this hypothesis and as further verification of these findings, we labeled XCL100- and XCL100(C260S)-injected oocytes with [32P]orthophosphate in the absence or presence of progesterone. The Myc-tagged proteins were then immunoprecipitated and analyzed by autoradiography. Figure 2C confirms that both XCL100 proteins were phosphorylated in response to progesterone treatment; again, the more slowly migrating forms of XCL100(C260S) were apparent. In addition, XCL100(C260S), and to a lesser extent XCL100, were phosphorylated in G2-arrested oocytes, as suggested by the above-mentioned experiments with AIP (Figure 2B).

To identify the residue(s) phosphorylated within XCL100, we excised the radiolabeled XCL100 bands and performed phosphoamino acid analysis. Excised proteins were subjected to acid hydrolysis followed by two-dimensional thin-layer electrophoresis. The results (Figure 2D) demonstrate that in G2-phase oocytes, both XCL100 and XCL100(C260S) were phosphorylated on one or more serine residues; in response to progesterone, an additional spot of threonine phosphorylation appeared in each sample. The phosphothreonine present in these samples was not due to contamination from coimmunoprecipitating p42 MAPK (our unpublished observations). Thus, XCL100 is differentially phosphorylated in the presence and absence of progesterone, on one or more serine residues during G2 phase, and on both serine and threonine during M phase.

**XCL100 Phosphorylation Requires p42 MAPK Activity**

Presuming that the only intrinsic difference between the XCL100 and XCL100(C260S) proteins is their catalytic activity, these results suggested two main possibilities: either XCL100 undergoes autodephosphorylation, or the M-phase phosphorylation of XCL100 is a p42 MAPK-dependent process. To further investigate the latter possibility, we examined whether XCL100 phosphorylation parallels that of p42 MAPK in vivo. If XCL100 phosphorylation were continuously dependent on p42 MAPK activity throughout meiosis, for example, then dephosphorylation of the phosphatase would be predicted at approximately the same time as p42 MAPK inactivation in fertilized eggs. To circumvent the severe cell cycle-inhibitory effect and apparent instability of overexpressed XCL100 in progesterone-treated oocytes (Figures 1 and 2A), XCL100(C260S) was used in the following experiments. Oocytes microinjected with XCL100(C260S) mRNA were incubated with progesterone overnight, thereby allowing the oocytes to complete maturation and arrest the cell cycle at metaphase of meiosis 2 (cytostatic factor arrest). Mature oocytes were then incubated with the calcium ionophore A23187 to simulate fertilization (Sohaskey and Ferrell, 1999), and samples were collected at various times for analysis by SDS-PAGE and immunoblotting.

In A23187-treated oocytes, XCL100(C260S) phosphorylation and p42 MAPK activation persisted for 30–45 min, at
of 5 CL100(C260S) and incubated overnight in the presence or absence of Xcroinjected with mRNA (41 ng) encoding Myc-tagged that of p42 MAPK throughout meiosis. Stage VI oocytes were mi-

Figure 3. M-phase phosphorylation of XCL100(C260S) is p42 MAPK dependent. (A) XCL100(C260S) phosphorylation parallels that of p42 MAPK throughout meiosis. Stage VI oocytes were mi-

croinjected with mRNA (41 ng) encoding Myc-tagged XCL100(C260S) and incubated overnight in the presence or absence of 5 µg/ml progesterone. The next day, immature (G2) oocytes were collected immediately; mature oocytes (M) were transferred to OR2 containing 5 µM A23187, and collected at the indicated times. XCL100(C260S) was detected by 9E10 immunoblotting, after which CL100(C260S) was detected by 9E10 immu-

which time both proteins shifted to their more rapidly mi-

grating, dephosphorylated forms (Figure 3A). The highly phosphorylated form of XCL100(C260S) reappeared between 75 and 90 min, concomitant with the reactivation of p42 MAPK. Reactivation of p42 MAPK in these oocytes may reflect the restoration of metaphase arrest triggered by in-

completely degraded Mos protein, analogous to the arrest induced by microinjection of Mos into cleaving blastomeres (Sagata et al., 1989). Thus, the phosphorylation state of XCL100(C260S), and presumably by extension that of en-

dogenous XCL100, largely correlates with the activation state of p42 MAPK throughout the meiotic cell cycle.

To determine whether p42 MAPK activation is necessary for XCL100(C260S) phosphorylation, we exploited the pharmacological MEK inhibitor U0126 to block p42 MAPK activation in oocytes expressing the catalytically inactive phosphatase (Favata et al., 1998). As shown in Figure 3B, XCL100(C260S) was quantitatively phosphorylated in re-

sponse to progesterone treatment, an effect that was com-

pletely abrogated by the inclusion of 50 µM U0126. Signifi-

antly, no phosphorylation of XCL100(C260S) was detected in the presence of U0126 despite an approximately sevenfold activation of the MAPK family member JNK in these oocytes (our unpublished observations; Bagowska et al., 2001). Thus, U0126 treatment prevents p42 MAPK activation in oocytes while having little or no effect on JNK activation. Notably, p42 MAPK and JNK are the only MAPK family members known to be activated during Xenopus oocyte maturation, further suggesting that the observed effects of U0126 are attributable to its specific inhibition of the p42 MAPK cascade.

To confirm and extend these findings, we subjected XCL100(C260S)-injected oocytes to hyperosmolar stress by exposure to sorbitol, which activates JNK but not p42 MAPK (Bagowska et al., 2001). Consistent with the coincidence of p42 MAPK activation and XCL100 phosphorylation observed in previous experiments, neither p42 MAPK nor XCL100(C260S) was phosphorylated in oocytes incubated in 0.2 M sorbitol for 1 h, by which time JNK was robustly activated (Figure 3C). These data suggest that activation of p42 MAPK, but not the closely related MAPK family member JNK, is required for XCL100 phosphorylation in oocytes.

To address the question of whether p42 MAPK directly phosphorylates XCL100 in oocytes, we tested the ability of recombinant p42 MAPK to phosphorylate XCL100(C260S) in vitro by using XCL100(C260S) expressed in and immuno-

expressing XCL100(C260S) were left unstimulated for 5 h, stimu-

lated with 5 µg/ml progesterone for 5 h (GVBD$_{50}$ of ~3.5 h), or treated with 0.2 M sorbitol for 1 h. XCL100(C260S) and p42 MAPK were detected by immunoblotting with 9E10 (top) and DC3 (mid-

dle), respectively. JNK activity assays (five oocytes per sample) were performed as described (Bagowska et al., 2001), and phosphor-

ylated GST-Jun was visualized by autoradiography (bottom). (D) p42 MAPK phosphorylates XCL100(C260S) on serine and threonine in vitro. Lysates from G2-arrested oocytes expressing XCL100(C260S) were subjected to immunoprecipitation with 9E10 (top) and DC3 (mid-

dle). Immunoprecipitates were treated with (+) or without (−) active mouse p42 MAPK (2 U/µl) plus γ-32P-ATP for 30 min at 30°C and analyzed by autoradiography (top) and 9E10 immunoblotting (mid-

dle). Phosphoamino acid analysis of phosphorylated XCL100(C260S) (bottom) was performed as described in Figure 2D. XCL100(C260S)-P, phosphorylated XCL100(C260S).
precipitated from G2-arrested oocytes. Active p42 MAPK phosphorylated XCL100(C260S) on both serine and threonine residues, causing a modest reduction in its electrophoretic mobility (Figure 3D).

Taken together, these results indicate that p42 MAPK activation is a prerequisite for XCL100 phosphorylation at the G2-M transition of the meiotic cell cycle.

**Threonine Phosphorylation of XCL100 Maps to Threonine 168**

The discovery that threonine phosphorylation of XCL100 is progesterone-inducible suggested that this phosphorylation may play a particularly important role in the regulation of XCL100 function during meiosis. We therefore sought to map the site of threonine phosphorylation within XCL100. Given our observations that XCL100 phosphorylation is a p42 MAPK-dependent process and that p42 MAPK can phosphorylate XCL100 on both serine and threonine in vitro (Figure 3D), we reasoned that p42 MAPK itself may be the kinase responsible for XCL100 threonine phosphorylation. p42 MAPK phosphorylates substrates containing the motif X-X-(Ser/Thr)-Pro, where X represents any neutral or basic amino acid (Clark-Lewis et al., 1991; Gonzalez et al., 1991; Haycock et al., 1992; Corbalan-Garcia et al., 1996). We scanned the predicted protein sequence of XCL100 for p42 MAPK consensus phosphorylation sites and identified six such sites, four SP and two TP sites (our unpublished observations). Notably, the carboxy terminus of XCL100 also contains a Phe-Asn-Phe-Pro (FNFP) sequence motif, this motif has been shown to serve as a docking site through which p42 MAPK binds many of its substrates (Jacobs et al., 1999).

Only one of the two potential sites of p42 MAPK-mediated threonine phosphorylation, corresponding to threonine 168 in the *Xenopus* phosphatase, is invariant across several MKP family members (our unpublished observations). We mutated this threonine to either a nonphosphorylatable valine (T168V) or a potentially phosphomimetic glutamate (T168E) and repeated in vivo labeling experiments to assess the ability of our mutant XCL100 proteins to undergo threonine phosphorylation. Whereas progesterone stimulated the upward mobility shift of the XCL100(C260S) and XCL100(C260S/T168E) proteins on SDS-PAGE, it largely failed to do so for XCL100(C260S/T168V) (Figure 4A). This finding implicates phosphorylation of threonine 168 as a prerequisite for this mobility shift. Moreover, whereas XCL100(C260S) exhibited its characteristic serine and threonine phosphorylation in progesterone-treated oocytes, XCL100(C260S/T168V) and XCL100(C260S/T168E) were each phosphorylated exclusively on serine under identical circumstances (Figure 4B). Comparable results were obtained for the wild-type XCL100 protein (our unpublished observations). Mutation of threonine 168 affected neither the catalytic activity of the phosphatase (our unpublished observations) nor its ability to bind p42 MAPK in vivo (Figure 5B), suggesting that the lack of threonine phosphorylation in these proteins is not attributable to global changes in protein conformation. In addition, the observed reduction in the electrophoretic mobility of the XCL100(C260S/T168E) protein during M phase supports the notion that XCL100 is also phosphorylated on one or more serine residues at the G2-M transition. We conclude from these results that threonine 168 represents the lone site of XCL100 threonine phosphorylation in progesterone-stimulated oocytes.

**XCL100 Phosphorylation Does not Alter Its Catalytic Activity toward p42 MAPK**

Several protein phosphatases, including the dual-specificity phosphatase Cdc25C (Izumi et al., 1992; Kumagai and Dunphy, 1996), undergo a phosphorylation-induced change in catalytic activity. For this reason, we asked whether the progesterone-stimulated, p42 MAPK-dependent phosphorylation of XCL100 served to modulate its phosphatase activity. We found that by titrating the amount of XCL100 mRNA injected per oocyte, we were able to achieve a level of XCL100 expression (at 5 ng of mRNA injected) that was permissive for meiotic maturation (and hence p42 MAPK activation and XCL100 phosphorylation) yet at the same time sufficient for performing immunocomplex phosphatase assays.

Immature, G2-arrested oocytes were microinjected with 5 ng of XCL100 mRNA, incubated in the presence or absence of progesterone, and collected shortly after GVBD. Myc-tagged XCL100 proteins were then immunoprecipitated, and catalytic activity toward 32P-labeled Xenopus p42 MAPK(K57R) was measured in an immunocomplex phosphatase assay. As shown in Figure 5A (top), XCL100 was highly active toward bisphosphorylated p42 MAPK(K57R) regardless of the cell cycle status of the oocytes from which the protein was isolated. Phosphatase activity was not due to a lack of XCL100 phosphorylation because XCL100 isolated from progesterone-treated oocytes was quantitatively phosphorylated, as indicated by its characteristic mobility shift on SDS-PAGE (Figure 5A,
Phosphorylation frequently induces the association or dissociation of enzyme-substrate complexes. Given this prominent role for phosphorylation in influencing protein–protein interactions, we determined whether progesterone-stimulated phosphorylation of XCL100 on threonine 168 alters the affinity of the phosphatase for p42 MAPK in vivo. We repeated our communoprecipitation experiments from Figure 1B by using the XCL100(C260S/T166V) and XCL100(C260S/T168E) proteins, and immunoblotted for the presence of p42 MAPK in the immunoprecipitates. Comparable amounts of p42 MAPK coprecipitated with each of the XCL100(C260S) proteins from M-phase oocytes, whereas no p42 MAPK was detected in samples from G2-arrested oocytes (Figure 5B). Thus, the phosphorylation state of threonine 168 apparently does not influence the affinity of XCL100 for p42 MAPK in progesterone-treated oocytes. This conclusion is consistent with our finding that mutation of threonine 168 did not affect the potency of XCL100 in preventing GVBD (our unpublished observations). We cannot, however, strictly exclude the possibility that the substrate-trapping capacity of the XCL100(C260S) mutant masks an otherwise inhibitory effect of threonine 168 mutation on p42 MAPK binding.

**XCL100 Is a Highly Labile Protein in Sorbitol-treated Oocytes**

Previously, we noted that the expression levels of our Myc-tagged XCL100 protein were consistently and dramatically reduced in progesterone-stimulated oocytes relative to unstimulated oocytes (Figures 1B and 2A). This observation suggested that proteolysis may play an important role in regulating the levels of XCL100 during meiosis. Indeed, MKP-1, the murine homolog of XCL100, is a labile protein in mouse (Charles et al., 1992) and hamster (Brondello et al., 1999) fibroblasts, with an estimated half-life of 45 min.

To test the hypothesis that XCL100 is degraded in a progesterone-dependent manner, we treated XCL100-expressing oocytes 7 h after microinjection with progesterone in the presence of the protein synthesis inhibitor cycloheximide. Additionally, as a test of XCL100 stability in response to hyperosmolar stress, we exposed a subset of XCL100-injected oocytes to sorbitol in the presence of cycloheximide. Figure 6 demonstrates that XCL100 was a labile protein in all three sets of oocytes tested. Surprisingly, however, the rate of XCL100 degradation measured in sorbitol-treated oocytes (half-life of ~45 min for n = 6 experiments) was greater than twice that measured in control and progesterone-stimulated oocytes (half-life of 2–3 h for n ≤ 4 experiments). Degradation was specific for XCL100 and was not due to cycloheximide-induced cell toxicity, as the levels of endogenous MEK and p42 MAPK remained constant throughout the time course (Figure 6A). The apparent contradiction between the slow rate of progesterone-dependent XCL100 degradation seen in these experiments and the more rapid rate inferred from our previous experiments (Figures 1B and 2A) may be due, in part, to the inhibitory effect of cycloheximide on progesterone-induced maturation in *Xenopus* oocytes (see DISCUSSION). Based on these results, we conclude that hyperosmolar stress up-regulates the proteolysis of an already labile XCL100 protein.

**Threonine Phosphorylation of XCL100 Does not Alter Its Affinity for p42 MAPK**

Phosphorylation frequently induces the association or dissociation of enzyme-substrate complexes. Given this prominent role for phosphorylation in influencing protein–protein interactions, we determined whether progesterone-stimulated phosphorylation of XCL100 on threonine 168 alters the affinity of the phosphatase for p42 MAPK in vivo. We repeated our communoprecipitation experiments from Figure 1B by using the XCL100(C260S/T166V) and XCL100(C260S/T168E) proteins, and immunoblotted for the presence of p42 MAPK in the immunoprecipitates. Comparable amounts of p42 MAPK coprecipitated with each of the XCL100(C260S) proteins from M-phase oocytes, whereas no p42 MAPK was detected in samples from G2-arrested oocytes (Figure 5B). Thus, the phosphorylation state of threonine 168 apparently does not influence the affinity of XCL100 for p42 MAPK in progesterone-treated oocytes. This conclusion is consistent with our finding that mutation of threonine 168 did not affect the potency of XCL100 in preventing GVBD (our unpublished observations). We cannot, however, strictly exclude the possibility that the substrate-trapping capacity of the XCL100(C260S) mutant masks an otherwise inhibitory effect of threonine 168 mutation on p42 MAPK binding.

**Figure 5.** (A) Phosphorylation of XCL100 does not alter its phosphatase activity toward p42 MAPK. Stage VI oocytes were microinjected with mRNA (5 ng) encoding Myc-tagged XCL100 and incubated 2 h later in the absence (−) or presence (+) of 5 μg/ml progesterone. Oocytes (10 per sample) were collected after 7 h (−GVBD) and immediately lysed. XCL100 proteins were immunoprecipitated with 9E10, washed extensively, and their activity toward [32P]p42 MAPK(K57R) was measured in an immunocomplex phosphatase assay (top). Samples were subjected to SDS-PAGE and autoradiography, and 32P remaining in p42 MAPK(K57R) was quantified and normalized to control [32P]p42 MAPK(K57R) (represented as 100%). Data are from one experiment with assays performed in duplicate and are shown as averages ± ranges. Samples processed in parallel, from which the [32P]p42 MAPK(K57R) had been omitted, were immunoblotted with 9E10 followed by colorimetric detection to visualize the immunoprecipitated XCL100 proteins (bottom). (B) Threonine phosphorylation of XCL100(C260S) does not alter its affinity for active p42 MAPK in vivo. Stage VI oocytes were microinjected with mRNA (46 ng) encoding Myc-tagged XCL100(C260S) having its threonine 168 mutated to valine (T168V) or glutamate (T168E). Oocytes were treated Oocytes 7 h after microinjection with progesterone in the presence of the protein synthesis inhibitor cycloheximide. Additionally, as a test of XCL100 stability in response to hyperosmolar stress, we exposed a subset of XCL100-injected oocytes to sorbitol in the presence of cycloheximide. Figure 6 demonstrates that XCL100 was a labile protein in all three sets of oocytes tested. Surprisingly, however, the rate of XCL100 degradation measured in sorbitol-treated oocytes (half-life of ~45 min for n = 6 experiments) was greater than twice that measured in control and progesterone-stimulated oocytes (half-life of 2–3 h for n ≤ 4 experiments). Degradation was specific for XCL100 and was not due to cycloheximide-induced cell toxicity, as the levels of endogenous MEK and p42 MAPK remained constant throughout the time course (Figure 6A). The apparent contradiction between the slow rate of progesterone-dependent XCL100 degradation seen in these experiments and the more rapid rate inferred from our previous experiments (Figures 1B and 2A) may be due, in part, to the inhibitory effect of cycloheximide on progesterone-induced maturation in *Xenopus* oocytes (see DISCUSSION). Based on these results, we conclude that hyperosmolar stress up-regulates the proteolysis of an already labile XCL100 protein.
Increased Stability of XCL100 after Its p42 MAPK-dependent Phosphorylation in Sorbitol-treated Oocytes

At least two lines of evidence suggested that p42 MAPK-dependent phosphorylation of XCL100 may lead to a decrease in the rate of its degradation in vivo. First, as documented in Figure 1B, phosphatase-inactive XCL100(C260S) was consistently more stable in progesterone-treated oocytes than was the wild-type phosphatase, coincident with phosphorylation both followed estradiol treatment of XCL100 and G2-phase oocytes, respectively (Figure 5A, bottom). These findings implied a decrease in the rate of XCL100 degradation after its phosphorylation.

To investigate this possibility, we exploited an inducible activator of the p42 MAPK cascade, a chimeric protein comprising an oncogenic form of human c-Raf-1 (ΔRaf-1 DD) fused to the hormone-binding domain of the human estrogen receptor (Bosch et al., 1997). Stage VI oocytes were microinjected with in vitro-transcribed mRNA encoding this ΔRaf-DD:ER protein and incubated overnight before being injected again with XCL100 mRNA. Estradiol (2 μM) was added several hours later to trigger ΔRaf-DD:ER kinase activation, after which sorbitol and cycloheximide were added, and XCL100 degradation was assessed by SDS-PAGE and immunoblotting. Figure 7 shows that p42 MAPK activation and XCL100 phosphorylation both followed estradiol treatment of ΔRaf-DD:ER-expressing oocytes. Dramatically, the degradation rate of nonphosphorylated XCL100 (half-life of ~50 min) was nearly 3 times that of its phosphorylated counterpart (half-life of ~145 min). Stabilization of the XCL100 protein was not due to a nonspecific estradiol-mediated effect, because the rate of XCL100 degradation was unaffected by estradiol treatment in oocytes not expressing ΔRaf-DD:ER.
Collectively, these results indicate a role for p42 MAPK-mediated phosphorylation in stabilizing the XCL100 protein during meiosis.

**DISCUSSION**

Constitutive p42 MAPK activation can have dramatic biological consequences, including inappropriate cell fate changes, cellular senescence (Lin et al., 1998; Zhu et al., 1998), and cellular transformation (Cowley et al., 1994; Mansour et al., 1994). p42 MAPK activity must therefore be tightly controlled throughout the cell cycle to ensure the proper coordination of physiological events. Many dual-specificity phosphatases capable of inactivating MAPK family members in vivo have now been cloned and characterized from diverse model organisms. Little is known, however, regarding the mechanisms by which cells restrict the functions of these important proteins. Herein, we reveal one such mechanism for regulating a Xenopus MKP-1/CL100 homolog in progesterone- and sorbitol-treated oocytes. Our results elucidate, for the first time, the regulation of a MAPK phosphatase during the meiotic cell cycle and in response to cellular stress.

**Stabilization of XCL100 Follows Its p42 MAPK-dependent Phosphorylation**

Foremost among our findings is the discovery that stabilization of the normally labile XCL100 protein follows its p42 MAPK-dependent phosphorylation. Two suggestive pieces of evidence emerging from our initial studies prompted us to investigate this possibility directly. First, despite their comparable expression levels in G2-arrested oocytes, levels of wild-type XCL100 were markedly reduced relative to those of catalytically inactive XCL100(C260S) in progesterone-treated oocytes. This difference in expression levels correlated with M-phase phosphorylation of XCL100(C260S) but not XCL100, which in turn correlated with the suppression of progesterone-stimulated p42 MAPK activation by the wild-type phosphatase. Second, similar amounts of nonphosphorylated and phosphorylated XCL100 were isolated from G2-phase and M-phase oocytes, respectively, only when phosphatase expression levels were too low to inhibit p42 MAPK activation and GVBD (Figure 5A). Thus, indirect evidence suggested a cause-and-effect relationship between p42 MAPK-mediated phosphorylation and a decrease in the rate of XCL100 degradation in oocytes.

Direct testing of this hypothesis, however, proved difficult. This difficulty is due to similarly slow rates of XCL100 proteolysis in unstimulated and progesterone-stimulated oocytes treated with cycloheximide to measure protein degradation directly. We suspect that incubation of progesterone-treated oocytes in cycloheximide may increase the robustness of the p42 MAPK/XCL100 system. Nevertheless, in the oocyte this negative feedback loop appears to be overwhelmed by a stronger positive feedback system (Gotth et al., 1995; Matten et al., 1996; Roy et al., 1996; Howard et al., 1999; this study) that maintains p42 MAPK activity at high levels despite high levels of phosphatase activity (Sohaskey and Ferrell, 1999).

**XCL100 Stabilization Confers Negative Feedback upon p42 MAPK Cascade**

In G2-arrested oocytes, XCL100 degradation is slow and appears to be balanced by protein synthesis. Progesterone-triggered activation of the p42 MAPK cascade presumably overwhelms the activity of endogenous XCL100, leading to its p42 MAPK-dependent phosphorylation and stabilization. In contrast, exposure to sorbitol (and possibly other cellular stresses) causes rapid degradation of XCL100 in the absence of significant p42 MAPK activation. Additional work will be needed to determine whether this absence of p42 MAPK activation is due to a lack of cross-talk between the JNK and p42 MAPK pathways in sorbitol-exposed oocytes, or whether instead p42 MAPK activation is actively inhibited under these circumstances.

The fact that p42 MAPK stabilizes its inactivator, XCL100, means that the p42 MAPK/XCL100 system constitutes a negative feedback loop that should tend to moderate or temporally limit the activation of p42 MAPK. Moreover, p42 MAPK may positively regulate XCL100 through at least two other mechanisms: activation of p42 MAPK can induce expression of the closely related phosphatases MKP-1 and MKP-2 (Bromello et al., 1997), and, by analogy to other MKP family members, p42 MAPK may catalytically activate XCL100 by binding to its amino terminus (Campos et al., 1998; Dowd et al., 1998; Feldl et al., 2000; Chen et al., 2001; Slack et al., 2001). The presence of multiple activation mechanisms may increase the robustness of the p42 MAPK/XCL100 system. Nevertheless, in the oocyte this negative feedback loop appears to be overwhelmed by a stronger positive feedback system (Gotth et al., 1995; Matten et al., 1996; Roy et al., 1996; Howard et al., 1999; this study) that maintains p42 MAPK activity at high levels despite high levels of phosphatase activity (Sohaskey and Ferrell, 1999).

In contrast, the relationship between XCL100 and its substrate JNK appears to be fundamentally different. Whereas overexpressed XCL100 does prevent JNK activation in oocytes subjected to hyperosmolar stress (our unpublished observations), JNK activation does not suffice to bring about XCL100 phosphorylation and stabilization (Figure 3C).
Thus, the JNK/XCL100 system appears not to constitute a negative feedback loop. Moreover, because sorbitol strongly stimulates both JNK activation and degradation of the phosphatase then either JNK or something upstream of JNK appears to negatively regulate XCL100. This could represent a “double negative” feedback system, with XCL100 and JNK mutually inhibiting each other. Such a system can, in principle, exhibit switch-like bistable behavior, with a stable high JNK/low XCL100 state and a stable low JNK/high XCL100 state, but no stable intermediate states. Indeed, recent work has shown that JNK does exhibit bistable responses in oocytes (Bagowksi and Ferrell, 2001). It will be of interest to determine what contribution XCL100 makes to the bistable behavior of JNK.

Meiotic Phosphorylation of XCL100: More than a Stabilizing Influence?

The observed stabilization of XCL100 after its p42 MAPK-dependent phosphorylation in oocytes is consistent with the results of Brondello et al. (1999), who found that p42/p44 MAPK-dependent phosphorylation of MKP-1 reduced the rate of its degradation in Chinese hamster lung fibroblast (CCL39) cells. Whereas they identified the sites of p42/p44 MAPK-mediated phosphorylation in MKP-1 as two serine residues exclusively, only one of which is present in the Xenopus phosphatase, we have mapped the progesterone-stimulated phosphorylation of XCL100 to one threonine and one or more serines. Moreover, we find that substitution of glutamate for this well-conserved threonine (Thr 168) partially stabilizes XCL100 in response to sorbitol (our unpublished observations). This apparent discrepancy may reflect a legitimate biological difference between different cell types.

Additionally, it is tempting to speculate that threonine phosphorylation may confer an additional level of regulation upon the Xenopus phosphatase. In support of this possibility, phosphatase-inactive XCL100(C260S) gel-filters in a high molecular weight complex in G2-arrested oocytes but dissociates from this complex during M phase, concomitant with its progesterone-induced phosphorylation at the G2-M transition (our unpublished observations). It will be important to determine how distinct MKP family members are differentially regulated in diverse biological contexts.

We also noted serine phosphorylation of XCL100 in G2-arrested oocytes. The presence of eight consensus phosphorylation sites for PKA within XCL100 (our unpublished observations), together with the importance of PKA for maintaining oocytes in a G2-like arrest, suggest that phosphorylation by PKA may somehow regulate XCL100 function. Although the physiological relevance of this G2-phase phosphorylation is uncertain, one intriguing possibility arises from recent work by Rafael Pulido’s laboratory. This group showed that, in COS-7 cells, the tyrosine phosphatase PTP-SL inactivates and anchors p42 MAPK in the cytoplasm through association of p42 MAPK with a kinase interaction motif (KIM) in PTP-SL (Zúñiga et al., 1999). PKA-catalyzed phosphorylation of Ser 231, located within the KIM, abrogates p42 MAPK binding and favors nuclear translocation of the kinase (Blanco-Aparicio et al., 1999). Future work will seek to determine whether PKA-mediated phosphorylation regulates XCL100 function, perhaps by altering its affinity for either MAPK family members or the higher molecular weight complex described above.

XCL100 Functions as Both Enzyme and Substrate for p42 MAPK

Despite its ability to fully inactivate both p42 MAPK and JNK (Chu et al., 1996; Hirsch and Stork, 1997; our unpublished observations) in vivo, XCL100 appears to be phosphorylated by a strictly p42 MAPK-dependent mechanism. Thus, whereas XCL100 can act as both enzyme and substrate in its relationship with p42 MAPK, the phosphatase acts only as an enzyme in its association with the related MAPK family member JNK. This finding implies that the reciprocal reactions, dephosphorylation of p42 MAPK by XCL100 and phosphorylation of XCL100 by p42 MAPK, are likely mediated by distinct binding events between the two proteins. Consistent with this proposal, we noted the presence of an FNFP motif within the carboxy terminus of XCL100. Jacobs et al. (1999) demonstrated that FXFP is an evolutionarily conserved motif that mediates binding of p42 MAPK to various substrate proteins. The discovery that both substrate specificity and tight substrate binding are conferred by the amino terminus of the related phosphatase MKP-3 (Campe et al., 1998; Muda et al., 1998) supports the hypothesis that the FNFP motif within XCL100 mediates its role as a p42 MAPK substrate. In addition, the absence of an analogous experimentally defined JNK docking site (Yang et al., 1998a,b; Jacobs et al., 1999) in XCL100 is consistent with our conclusion that this phosphatase is a poor substrate for JNK.

XCL100 and Protein Degradation Machinery

Brondello et al. (1999) showed that MKP-1 degradation proceeds via the ubiquitin-directed proteasome complex. Degradation of XCL100 presumably proceeds via a similar mechanism, although this remains to be formally demonstrated. Moreover, important mechanistic details concerning the targeted degradation of XCL100 and MKP-1 remain a mystery, because neither of these proteins contains any of the recognizable destruction motifs typically found in other labile proteins, including PEST sequences (Rogers et al., 1986), a destruction box (Glotzer et al., 1991), or a KEN box (Pfeifer and Kirschner, 2000). Identification of the sequenc(e)s within XCL100 that direct its ubiquitin-dependent degradation will prove crucial in clarifying the role played by targeted degradation of MKP family members during the meiotic and mitotic cell cycles.

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