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The Casein Kinase II β Subunit Binds to Mos and Inhibits Mos Activity

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Mos is a germ cell-specific serine/threonine kinase and is required for *Xenopus* oocyte maturation. Active Mos stimulates a mitogen-activated protein kinase (MAPK) by directly phosphorylating and activating MAPK kinase (MKK). We report here that the *Xenopus* homolog of the β subunit of casein kinase II (CKIIβ) binds to and regulates Mos. The Mos-interacting region of CKIIβ was mapped to the C terminus. Mos bound to CKIIβ in somatic cells ectopically expressing Mos and CKIIβ as well as in unfertilized *Xenopus* eggs. CKIIβ inhibited Mos-mediated MAPK activation in rabbit reticulocyte lysates and repressed MKK activation by v-Mos in a coupled kinase assay. In addition, microinjection of CKIIβ mRNA into *Xenopus* oocytes inhibited progesterone-induced meiotic maturation and MAPK activation, presumably by binding of CKIIβ to Mos and thereby inhibiting MAPK activation. Moreover, this inhibitory phenotype could be rescued by another protein that binds to CKIIβ, CKIIα. The ability of ectopic CKIIβ to inhibit meiotic maturation and the detection of a complex between endogenous Mos and CKIIβ suggest that CKIIβ may act as an inhibitor of Mos during oocyte maturation, perhaps setting a threshold beyond which Mos protein must accumulate before it can activate the MAPK pathway.

Mos is a germ cell-specific serine/threonine kinase and is present at a very low level in fully grown (stage VI) *Xenopus* oocytes (46, 53, 55, 65). During oocyte maturation, *mos* translation is activated by progesterone secreted from the follicle cells (64, 65). Oocytes are then released from the prophase arrest of meiosis I and undergo a sequence of events including nuclear envelope breakdown (commonly known as germinal vesicle breakdown [GVBD]), completion of meiosis I, extrusion of the first polar body, meiosis II entry, and subsequent metaphase II arrest as unfertilized eggs (reviewed in reference 45). Upon fertilization, Mos protein is degraded and does not reappear during embryonic development (39, 80).

Mos is essential for progesterone-induced oocyte maturation. Microinjection of antisense *mos* oligonucleotides into *Xenopus* oocytes blocks progesterone-induced GVBD and the activation of maturation-promoting factor (MPF) (31, 65). MPF is a universal M-phase regulator that drives meiotic progression and consists of the $p34^{cdc2}$ protein kinase and the cyclin B protein (reviewed in references 32 and 49). In the absence of progesterone, injected Mos protein or *mos* mRNA can activate MPF and induce GVBD (64, 65, 82). These results suggest that Mos is necessary and sufficient to initiate meiosis. Mos is also needed for the later stage of oocyte maturation. Extracts of unfertilized eggs in meiosis II contain an activity, cytostatic factor (CSF), that causes metaphase II arrest and can be assayed by the induction of cleavage arrest when injected into blastomeres of dividing embryos (45). Mos has CSF activity, and immunodepletion of Mos from unfertilized *Xenopus* egg extracts abolishes CSF (15, 66, 82). Mos is also important for the meiosis II arrest in mice. Female mice lacking c-*mos* have reduced fertility and develop ovarian cysts or teratomas, since their oocytes fail to arrest at metaphase II and undergo parthenogenetic activation (13, 29).

The biological functions of Mos correlate well with the activation of a mitogen-activated protein kinase (MAPK; also known as ERK). MAPKs are implicated in many signaling processes (reviewed in references 12, 14, 44, 56, 67, and 79). MAPK and Mos are activated with similar kinetics in response to progesterone during oocyte maturation in *Xenopus* oocytes. While MPF activity drops between meiosis I and meiosis II, Mos and MAPK maintain high activities through meiosis and becomes inactive shortly after fertilization, when Mos protein is degraded (19, 25, 59). The activation of MAPK explains many biological effects of Mos. Constitutively active (thiophosphorylated) MAPK is able to induce GVBD in oocytes provided that protein synthesis is permitted (27). Like Mos, thiophosphorylated MAPK arrests mitosis at metaphase (28).

Several lines of evidence indicate that Mos activates MAPK by phosphorylating MAPK kinase (MKK or MEK). First, Mos phosphorylates and activates MKK in a partially purified system in vitro (60). Viral Mos (v-Mos) immunoprecipitates phosphorylate MKK at Ser-218 and Ser-222, the same residues phosphorylated by Raf, another MKK activator (62). Phosphorylation on both sites stimulates MKK activity (1, 24, 36, 42, 83). Second, Mos associates with MKK in a yeast two-hybrid test, while a mutant of Mos that does not interact with MKK in the two-hybrid system fails to activate MKK or MAPK (10). Third, microinjection of an anti-MKK antibody into *Xenopus* oocytes can block progesterone- or Mos-induced MAPK activation and GVBD (33). Last, another MKK activator, Raf, is not needed for Mos-induced MAPK activation in *Xenopus* oocyte extracts (70). These experiments suggest that MKK is directly activated by Mos. Once MKK is activated, it activates MAPK by phosphorylating MAPK on a threonine residue and a tyrosine residue (reviewed in references 12, 56, and 75).

Aside from the fact that Mos synthesis is controlled at the translational level (6, 21, 69), little is known about Mos regulation. To search for proteins that might regulate Mos activity,

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we used the yeast two-hybrid system and found that the casein kinase II (CKII) β subunit (CKIIβ) binds to Mos. We provide evidence that CKIIβ is a potential inhibitor of Mos. Our results suggest that CKIIβ may play a role in the Mos/MAPK signaling pathway.

MATERIALS AND METHODS

Yeast strains. The two-hybrid screen was carried out in *Saccharomyces cerevisiae* HF7c [*MAT*a *ura3-52 his3-200 lys2-801 ade2-101 trpl-901 leu2-3,112 gal4-542 gal80-538 LYS2::GAL1-HIS3 URA3::*(*GAL4*17-mer)$_3$-*CYCl-lacZ*] (17). Two reporter genes, *HIS3* and *lacZ*, were used for assaying interactions. Positive protein interactions were detected by the ability of yeast transformants to grow on synthetic medium lacking histidine, leucine, and tryptophan as well as by a β-galactosidase filter assay. Mating assays were performed in strain Y187 (*MAT*α *gal4 gal80 his3 trpl-901 ade2-101 ura3-52 leu2-3112 met URA3::GAL-lacZ*). Strain L40 [*MAT*a*his*Δ*200 trpl-901 leu2-3,112 ade2 LYS2::*(*lexAop*)$_4$-*HIS3 URA3::*(*lexAop*)$_8$-*lacZ*] was used for testing the specificity of the interactions (10, 77).

Plasmid construction. The full-length cDNA clone of *Xenopus mos* was subcloned into a GAL4 DNA-binding domain fusion vector pGBT9, using *Eco*RI/*Bam*HI sites (4). To subclone CKIIβ$_{18\text{-}215}$ into pVP16 or Myc-tagged fusion vector pCS3-MT (pCS) (76, 77), pGAD10-CKIIβ$_{18\text{-}215}$ was digested with *Bgl*II. The digestion product containing the CKIIβ$_{18\text{-}215}$ fragment with extra 5′ 39 bp derived from pGAD10 linker sequence was inserted into the *Bam*HI site of pVP16 or the *Bgl*II site of pCS. A 642-bp fragment of the full-length human CKIIβ and a 1,170-bp fragment of the full-length human CKIIα were amplified by PCR, introducing *Bam*HI sites 5′ and 3′ of the coding region for each DNA (the first initiation codon ATG was eliminated in constructing the fusions). The amplified fragments were subcloned into the *Bam*HI site of pVP16 or the *Bgl*II site of pCS. N-terminal and C-terminal deletion mutants of CKIIβ were generated by PCR using oligonucleotide primers containing *Bam*HI sites. The sequences of all amplified fragments were confirmed and were subsequently inserted into the *Bam*HI site of pGEX-2T to be expressed as glutathine *S*-transferase (GST) fusion proteins in *Escherichia coli*. A fragment encoding CKIIβ$_{1\text{-}160}$ was also subcloned into pCS in the same manner as the full-length CKIIβ. To construct pEBG-Mos, pKS$^+$-Mos was digested with *Bam*HI and *Kpn*I. The resulting fragment containing the full-length sequence of *Xenopus mos* cDNA was subcloned into the *Bam*HI/*Kpn*I site of pEBG-2T to be expressed in mammalian cells as a GST fusion (47, 48).

Library screening and sequencing. A cDNA library of *Xenopus laevis* oocytes in pGAD10 (Clontech) was used for the two-hybrid screen (4, 20). Yeast strain HF7c containing pGBT-Mos was transformed with the library plasmids, and 5×10^6 transformants were screened. After 5 days of growth on synthetic medium lacking histidine, leucine, and tryptophan, 65 transformants were His$^+$. His$^+$ colonies were subjected to the β-galactosidase assay; 22 were found to be both His$^+$ and LacZ$^+$. These colonies were grown on synthetic liquid medium lacking leucine to segregate plasmid pGBT9-Mos. The Leu$^+$ Trp$^-$ isolates were mated with yeast strain Y187 containing pGBT-Mos, pAS1-lamin, and pAS1-Cdk2. Of the 22, only 1 diploid was LacZ$^+$ in a pGBT9-Mos-specific manner. This GAL4 activation domain hybrid plasmid was isolated and sequenced by Taq DyeDeoxy terminator cycle sequencing, using a 5′ primer (5′-TACCACTACAATGGATG-3′) or a 3′ primer (5′-TCAACTTCACTTGAACGCCCC-3′) which hybridized with the pGAD10 vector. This clone was identified as CKIIβ$_{18\text{-}215}$. CKIIβ$_{18\text{-}215}$ and the full-length clones of human CKIIβ and CKIIα were subcloned into pVP16 as described above. These VP16 hybrid plasmids were then introduced into yeast strain L40 with the LexA fusion plasmids for testing the specificity of the interactions.

In vitro binding assay. pCS-CKIIβ$_{18\text{-}215}$, pCS-CKIIβ, and pCS-CKIIα plasmid DNAs were added to an in vitro transcription-translation system (Promega TNT) to be transcribed from the SP6 promoter and expressed as Myc-tagged proteins. The reactions were carried out in reticulocyte lysates for 2 h in the presence of [^{35}S]methionine (final concentration, 0.8 μCi/μl). A sample of 1 μl of reaction mixture was incubated with 1 μg of purified maltose-binding protein (MBP) or MBP-Mos from *E. coli* in 0.5 ml of 0.1% Triton immunoprecipitation (IP) buffer (0.1% Triton, 10 mM HEPES [pH 7.5], 2 mM EDTA, 150 mM NaCl, 50 mM NaF, 0.1% β-mercaptoethanol, 1 mM aprotinin, 1 mM phenylmethylsulfonyl fluoride) at 4°C for 1 h followed by the addition of 20 μl of amylose resin (New England Biolabs). After 4 h of incubation at 4°C, the resin was pelleted by brief centrifugation, washed three times with 0.1% Triton IP buffer containing 750 mM NaCl, and washed once with PAN [100 mM NaCl, 10 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES; pH 7.0), 1 mM aprotinin]. The bound proteins were eluted in sample buffer, resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and subjected to autoradiography.

To dissect the binding region of CKIIβ to Mos, N-terminal and C-terminal deletion mutants of GST-CKIIβ were purified from *E. coli*. A sample of 1.5 μl of in vitro transcription-translation mix containing [^{35}S]Mos (from pKS$^+$-Mos) was incubated in 0.5 ml of 0.1% Nonidet P-40 IP buffer with 2 μg of glutathione-Sepharose-bound GST or GST-CKIIβ deletion proteins. After incubation at 4°C for 1 h, the resin was washed three times with 0.1% Triton IP buffer containing 1 M NaCl, and the bound [^{35}S]Mos was eluted in sample buffer and resolved by SDS-PAGE.

Transient-transfection and infection assays. 293T cells were transiently cotransfected with pEBG or pEBG-Mos and pCS-CKIIβ$_{18\text{-}215}$ or pCS-CKIIβ, using a calcium phosphate method. Three days later, cells expressing both GST and Myc fusion proteins were harvested in 0.1% Triton IP buffer, vortexed, and centrifuged at 4°C and 8,000 × *g* for 15 min. Glutathione-Sepharose (New England Biolabs) was added to the supernatant. The resin was washed three times, using the same buffer with 200 mM NaCl added. The amounts of GST and GST-Mos were analyzed by using antibodies to GST. GST- or GST-Mos-associated Myc-tagged proteins were analyzed by anti-Myc immunoblotting.

Moloney murine sarcoma virus (Mo-MuSV-124) was collected from a subconfluent 10-cm-diameter dish of virus producer cell line (3). A 50% confluent 10-cm-diameter plate of NIH 3T3 cells was infected with Mo-MuSV-124 in the presence of Polybrene (10 μg/ml) for 10 h (46). Virus was removed, and fresh Dulbecco modified Eagle medium containing 10% fetal calf serum was added to the cells. The infected cells were harvested 40 h later. Cells were scraped into 1 ml of 1% Triton IP buffer containing 0.5 mM Na_3VO_4 and 2.5 μM microcystin. After vortexing, cell lysates were centrifuged at 4°C and 8,000 × *g* for 15 min. The supernatants were used for v-Mos immunoblotting and immunoprecipitation.

Immunoblotting and immunoprecipitation. 9E10 hybridoma supernatant was used for immunoblotting Myc-tagged proteins from 293T cells or *Xenopus* oocytes. For MAPK immunoblots, 10 μl of in vitro transcription-translation lysate containing *mos* DNA and [^{35}S]methionine was incubated with 0.2 μg of either GST or GST-CKIIβ. At different time intervals of Mos translation, 1 μl of lysate was removed and resolved by SDS-PAGE. A polyclonal antibody (1913.3) that recognizes *Xenopus* p42 MAPK was used for detecting the band shifts of endogenous rabbit ERK1 and ERK2 (58). In a separate experiment, purified GST-CKIIβ$_{1\text{-}160}$ was added to the in vitro transcription-translation lysates in the presence or absence of *mos* DNA. Two hours later, 1 μl of rabbit reticulocyte lysate was removed and analyzed by anti-MAPK immunoblotting. 1913.3 was also used for analyzing MAPK band shifts in *Xenopus* oocyte extracts. Extracts made from individual oocytes were resolved by SDS-PAGE and analyzed by immunoblotting.

Rabbit polyclonal anti-*Xenopus* Mos antibody K2 was used for c-Mos immunoblotting in *Xenopus* oocytes (51). The same antibody was used for *Xenopus* Mos immunoprecipitation. An anti-human CKIIβ antibody was used for CKIIβ immunoprecipitation (37). As a control, a preimmune antibody of CKIIβ was used for immunoprecipitation in oocytes and eggs. Extracts of 15 stage VI oocytes were prepared in 10 volumes of homogenization buffer by centrifugation at 15,000 × *g* for 20 min at 4°C (23). Supernatants were precleared with protein A-Sepharose (Sigma), mixed with 1.5 μl of anti-*Xenopus* Mos antibody or 4 μl of anti-CKIIβ antibody, and incubated in 0.5 ml of homogenization buffer at 4°C for 2 h. Then 20 μl of protein A-Sepharose were added, and incubation continued at 4°C for 1 h. The immune complex was then washed once with 10% sucrose–0.1% Triton IP buffer, three times with 0.1% Triton IP buffer, and once with PAN and analyzed by immunoblotting. In a separate experiment, a c-Mos antibody (Santa Cruz Biotechnology) was used to immunoprecipitate Mos in Myc-CKIIβ RNA-injected oocytes followed by 9E10 immunoblotting. v-Mos was immunoprecipitated from 0.5 ml of infected NIH 3T3 cell lysates, using 5 μl of anti-v-Mos antibody 37-55 (72). The same antibody preincubated with excess cognate peptide was used as a control. The v-Mos immunoprecipitation procedure was the same as that for c-Mos except that formaldehyde-fixed *Staphylococcus aureus* was used to recover the immune complex for the kinase assay (59).

Kinase assays. v-Mos kinase assays were performed in 25 μl of kinase buffer (150 mM NaCl, 10 mM HEPES [pH 7.4], 15 mM $MnCl_2$, 10 mM $MgCl_2$, 2 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride 1 mM aprotinin, 0.5 mM Na_3VO_4, 0.25 μM microcystin) containing 5 μM [γ-^{32}P]ATP (8,000 cpm/pmol), 1.5 μg of GST-CKIIβ, 1 μg of GST-MKK, and 2 μg of His$_6$-ERK2^{K52R} as needed. The reaction mix was incubated at 25°C for 15 min followed by 30°C for 10 min. Samples were resolved by SDS-PAGE and analyzed by autoradiography. CKII activity was assayed by monitoring the incorporation of [γ-^{32}P]ATP into a synthetic peptide substrate (RRRDDDSDDD) (38). The reaction was carried out in 25 μl of buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 20 mM $MgCl_2$, 10 μM [γ-^{32}P]ATP (2,000 cpm/pmol), and 6 μl of oocyte lysates. The reaction was stopped by spotting 20 μl of the reaction mix onto P81 phosphocellulose paper (Whatman). The assay was done in duplicate in the presence or absence of 0.1 mM substrate peptide to calculate the specific activity of CKII.

Microinjection of RNA and GVBD assay in *Xenopus* oocytes. Fully grown (stage VI) *Xenopus* oocytes were isolated from ovaries of female *Xenopus* by using collagenase treatment. Oocytes were cultured in 1× MBS (88 mM NaCl, 1 mM KCl, 0.7 mM $CaCl_2$, 1 mM $MgSO_4$, 5 mM HEPES [pH 7.8], 2.5 mM $NaHCO_3$, 10 μg of gentamicin per ml) at 16°C overnight prior to microinjection (26). 5′-capped RNAs of Myc-CKIIβ, Myc-CKIIβ$_{1\text{-}160}$, Myc-CKIIβ$_{18\text{-}215}$, and Myc-CKIIα were in vitro transcribed from pCS vector by using SP6 polymerase (76). Thirty nanoliters of in vitro-synthesized CKIIβ RNA was injected into each oocyte. In the coinjection experiment, in vitro-transcribed CKIIα RNA was mixed with CKIIβ RNA, and approximately 40 nl of RNA was microinjected into each oocyte. Three to four hours later, the injected oocytes were treated with progesterone for 10 to 12 h. GVBD was scored by the appearance of a white spot in the animal pole of an oocyte followed by fixation in 5% trichloroacetic acid and dissection. Oocyte extracts were normally prepared in 10 μl of 1% Triton IP buffer (including 0.5 mM Na_3VO_4 and 2.5 μM microcystin) per oocyte. The oocyte lysates were centrifuged at 4°C and 8,000 × *g* for 10 min.

TABLE 1. CKIIβ interacts with Mos in the yeast two-hybrid system[a]

VP16 fusion	Phenotype of indicated LexA fusion				
	Mos	CKIIα	c-Raf	MKK1	Lamin
$CKII\beta_{18-215}$	+	+	−	−	−
CKIIβ	+	+	−	−	−
$CKII\beta_{1-160}$	−	−	−	−	−
CKIIα	−	−	−	−	−
None	−	−	−	−	−

[a] Fusion constructs with either a LexA DNA-binding domain or a VP16 transactivation domain were transformed into yeast strain L40. Two-hybrid interaction was tested by β-galactosidase activity and yeast growth on medium lacking histidine. +, $LacZ^+$ His^+; −, $LacZ^-$ His^-. The LexA fusions of c-Raf and MKK1 were functional in binding to other VP16 fusions (10).

RESULTS

Interaction of CKIIβ with Mos in yeast. To search for Mos-interacting proteins, we performed a yeast two-hybrid screen using a *Xenopus* oocyte cDNA library and *Xenopus* Mos as bait. Of the 5 million yeast transformants screened, 22 expressed both *HIS3* and *lacZ* reporter genes, indicating the presence of putative Mos-interacting proteins. Among these 22 clones, only one interacted with Mos but not other proteins tested. Sequence analysis showed that this clone, $CKII\beta_{18-215}$, was the β subunit of CKII with deletion of 17 residues at the N terminus.

To test the specificity of CKIIβ binding to Mos, $CKII\beta_{18-215}$ was subcloned into a vector containing a VP16 activation domain (pVP16-$CKII\beta_{18-215}$). In addition, the full-length cDNA clone of human CKIIβ was subcloned into the same vector. The amino acid sequences of human and *Xenopus* CKIIβ are identical with the exception of one amino acid. Since this amino acid is not conserved in CKIIβ from other species, we assumed that human CKIIβ would behave in the same way as its *Xenopus* homolog. Various VP16 hybrid plasmids and LexA hybrid plasmids were introduced into yeast strain L40. The interaction of two proteins was assayed by the expression of two reporter genes, *HIS3* and *lacZ*. Human CKIIα was used as a control since it is known that CKIIβ binds to the α subunit in the CKII holoenzyme (22). As shown in Table 1, $CKII\beta_{18-215}$ and the full-length human CKIIβ interacted with Mos and CKIIα. The binding of $CKII\beta_{18-215}$ or CKIIβ to Mos was specific since neither one interacted with c-Raf, MKK1, or lamin (Table 1).

CKIIβ binds to Mos both in vitro and in vivo. To verify the yeast two-hybrid result, [^{35}S]methionine-labeled Myc-tagged $CKII\beta_{18-215}$, CKIIβ, and CKIIα, were prepared by in vitro translation in rabbit reticulocyte lysates. The lysates were then incubated with MBP-Mos and purified by using amylose resin. After several washes, bound proteins were detected by gel electrophoresis and autoradiography (Fig. 1A). Both Myc-CKIIβ and Myc-$CKII\beta_{18-215}$ bound to MBP-Mos. By contrast, no interaction of Myc-CKIIα with Mos was detected.

Next, we used the deletion mutants of CKIIβ to map the Mos-binding region. Interaction of CKIIα with CKIIβ requires the C-terminal residues (35, 43). The deletion of 55 amino acids from the C terminus of CKIIβ ($CKII\beta_{1-160}$) abolished CKIIβ binding to Mos as well as CKIIα (Table 1), suggesting that the C terminus of CKIIβ binds to both proteins. When the C-terminal fragments of CKIIβ were expressed as GST fusion protein in *E. coli* and tested for Mos binding, GST-$CKII\beta_{141-215}$ had the highest affinity for Mos (Fig. 1B). Therefore, the C terminus of CKIIβ is necessary and sufficient for mediating the Mos-CKIIβ interaction.

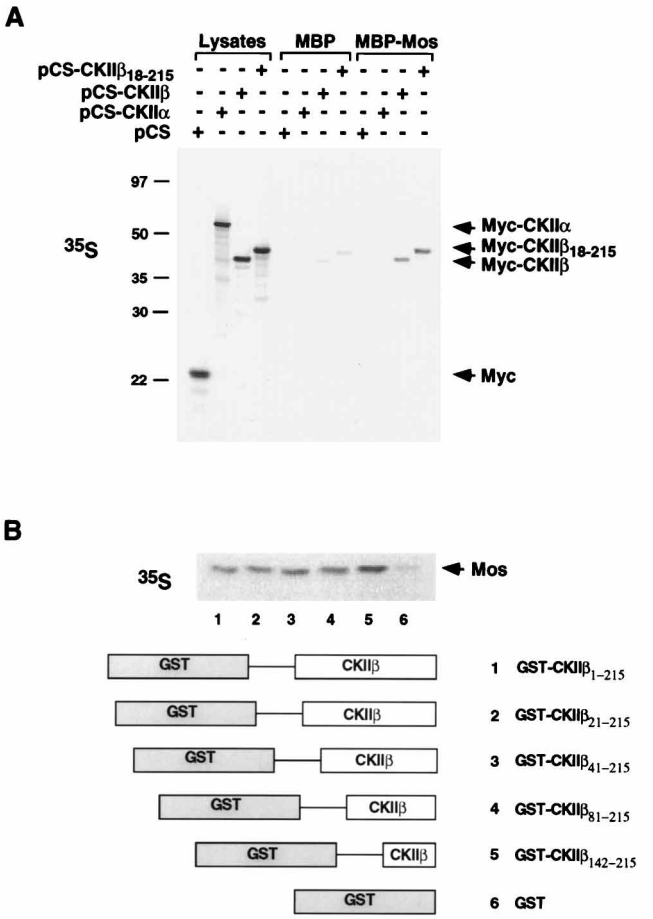

FIG. 1. In vitro binding of CKIIβ to Mos. (A) *Xenopus* $CKII\beta_{18-215}$ and the full-length human CKIIβ and CKIIα cDNAs were in vitro translated as Myc-tagged proteins in rabbit reticulocyte lysates. The lysates were then mixed with the bacterially purified MBP or MBP-Mos and purified with amylose resin. The binding of ^{35}S-labeled proteins was detected by autoradiography. Myc-$CKII\beta_{18-215}$ had a lower gel mobility than Myc-CKIIβ. The 13-residue 5′ linker sequences derived from pGAD10 in the original two-hybrid clone might contribute to its protein mobility on SDS-PAGE. (B) The full-length GST-CKIIβ and the N-terminal deletion mutant proteins of GST-CKIIβ were purified from *E. coli*, mixed with in vitro-translated [^{35}S]Mos, and purified on glutathione-Sepharose. The bound Mos was detected by autoradiography. Sizes are indicated in kilodaltons.

The demonstration of CKIIβ binding to Mos in a yeast two-hybrid screen and in vitro suggested that they might form a complex in cells. To test this possibility, 293T cells were transiently cotransfected with plasmids expressing GST-Mos and Myc-tagged CKIIβ or $CKII\beta_{18-215}$. Both GST-Mos and GST were expressed and purified on glutathione beads (Fig. 2A). Myc-CKIIβ and Myc-$CKII\beta_{18-215}$ were copurified with GST-Mos but not GST (Fig. 2B). Thus, CKIIβ is able to form a complex with Mos in somatic cells. Furthermore, we were able to detect the association of endogenous CKIIβ with Mos in *Xenopus* oocytes (see below).

CKIIβ inhibits Mos-mediated MKK and MAPK activation. We used two in vitro assays to study the effects of CKIIβ on Mos-mediated MKK and MAPK activation. In the first assay, the translation of *mos* mRNA in rabbit reticulocyte lysates induces the phosphorylation and activation of endogenous MAPK family members, ERK1 and ERK2, detected by their

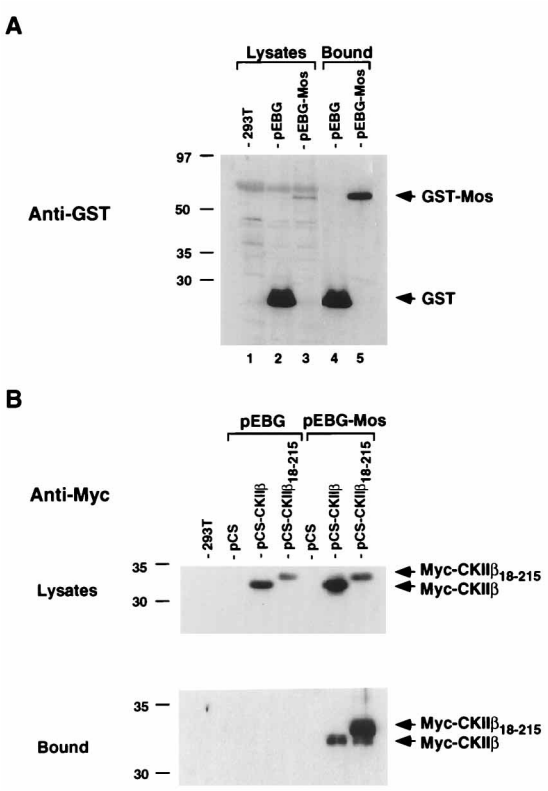

FIG. 2. Association of CKIIβ with Mos in 293T cells. 293T cells were transiently cotransfected with various pCS plasmids and pEBG plasmids. (A) GST and GST-Mos were expressed in 293T cells as detected by anti-GST immunoblotting and purified on glutathione-Sepharose. (B) Binding of Myc-CKIIβ or Myc-CKIIβ$_{18-215}$ to the GST fusion proteins was detected by anti-Myc immunoblotting. Sizes are indicated in kilodaltons.

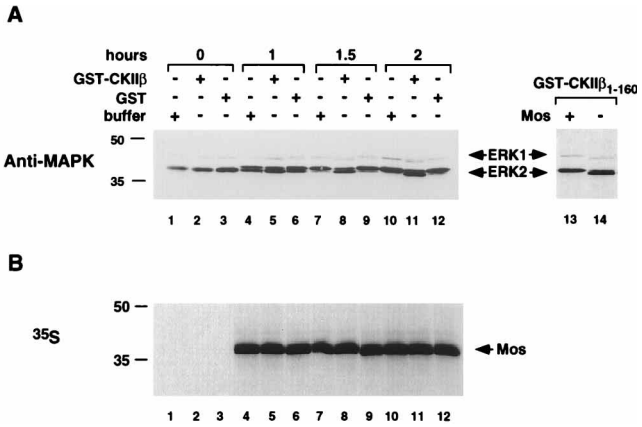

FIG. 3. Inhibitory effect of CKIIβ on Mos-mediated MAPK phosphorylation. (A) Mos protein was synthesized in rabbit reticulocyte lysates containing buffer, GST, and GST-CKIIβ. Aliquots were removed at various time intervals during Mos synthesis. ERK1 and ERK2 phosphorylation was analyzed by detecting electrophoretic mobility shifts on an anti-MAPK immunoblot (lanes 1 to 12). In a separate experiment, GST-CKIIβ$_{1-160}$ was added to a Mos in vitro translation system. After 2 h, aliquots were removed and ERK1 and ERK2 band shifts were detected (lanes 13 and 14). (B) In vitro-translated Mos from the used for panel A lysates was detected by autoradiography. Sizes are indicated in kilodaltons.

reduced electrophoretic mobilities on SDS-PAGE (bandshifts) (10, 71). GST-CKIIβ was added to rabbit reticulocyte lysates in which Mos protein was translated. Samples were removed during the course of Mos translation and assayed for ERK1 and ERK2 band shifts by anti-MAPK immunoblotting (10). Although the anti-MAPK antibody (1913.3) recognized ERK2 much better than ERK1, the two ERKs were phosphorylated similarly (58). Mos protein was translated, and partial ERK1 and ERK2 band shifts were observed after 1 h of incubation (Fig. 3A, lane 4). The phosphorylation of ERK1 and ERK2 was complete by 1.5 h in control lysates containing buffer or GST (Fig. 3A, lane 7 and 9). However, only partial ERK1 and ERK2 band shifts were detected in lysates containing GST-CKIIβ, even after 2 h of incubation (Fig. 3A, lane 11). The reduction in ERK phophorylation caused by GST-CKIIβ was not due to a decrease in Mos translation (Fig. 3B). Furthermore, a mutant of CKIIβ that does not bind Mos, GST-CKIIβ$_{1-160}$, did not inhibit MAPK phosphorylation induced by Mos (Fig. 3A, lane 13). Therefore, CKIIβ inhibits Mos-mediated MAPK phosphorylation most likely by interacting with Mos.

To directly assay the effect of CKIIβ on Mos kinase activity, v-Mos, which is a more active kinase than *Xenopus* Mos, was used. NIH 3T3 cells were infected with Mo-MuSV-124 containing v-Mos. Infected cells were morphologically transformed and expressed v-Mos protein (Fig. 4A). v-Mos was immunoprecipitated from these cells. Control immunoprecipitates were prepared with an anti-v-Mos antibody that had been blocked with excess peptide antigen. Immunoprecipitates were then incubated with GST-MKK, GST-CKIIβ, and an MKK substrate, recombinant ERK2^{K52R} (rERK2^{K52R}, which contains a mutation to make it kinase inactive) in the presence of radioactive ATP. Autophosphorylation of v-Mos was detected only in immunoprecipitates prepared in the absence of the

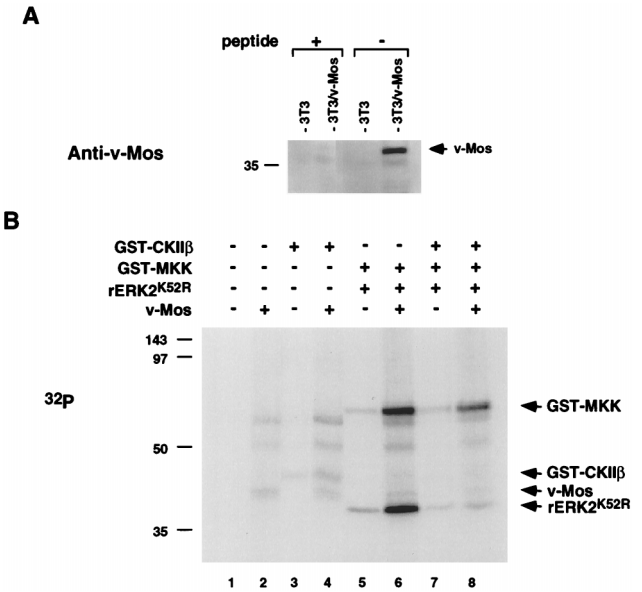

FIG. 4. Effect of CKIIβ on v-Mos kinase activity. NIH 3T3 cells were infected with Mo-MuSV-124. (A) v-Mos expression from infected cells was analyzed on an anti-v-Mos immunoblot. The antibody could be blocked by the addition of excess peptide antigen. (B) Purified GST-CKIIβ, GST-MKK, and His-tagged kinase-inactive mutant ERK2 (rERK2^{K52R}) were added as indicated to the controls or v-Mos immune complexes and incubated with [γ^{32}-P]ATP to allow protein phosphorylation. Samples were resolved by SDS-PAGE and analyzed by autoradiography. Immunoprecipitates prepared with anti-v-Mos antibody that had been preincubated with the excess peptide antigen were used as controls (lanes 1, 3, 5, and 7). Sizes are indicated in kilodaltons.

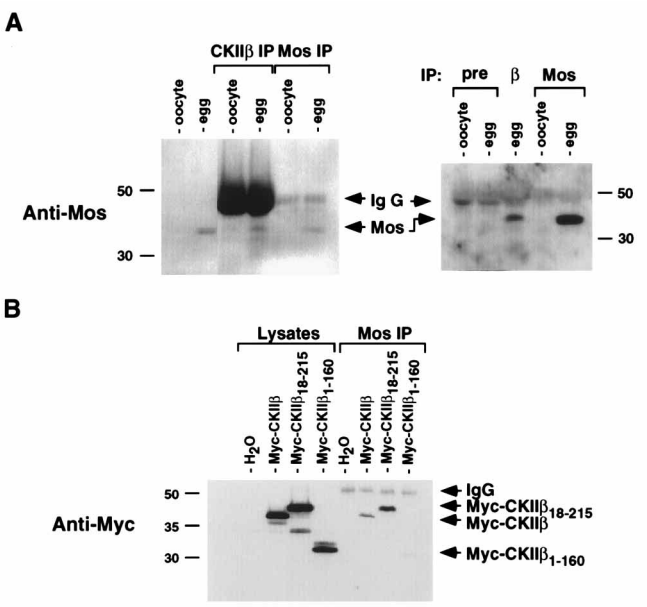

FIG. 5. Binding of CKIIβ to Mos in *Xenopus* oocytes. (A) Endogenous CKIIβ was immunoprecipitated (IP) from the stage VI oocytes or progesterone-induced mature eggs by using an antipeptide antibody and then immunoblotted with anti-Mos antibody K2. Mos immunoprecipitates obtained by using K2 antibody served as controls. In a separate experiment, different antibodies including a preimmune CKIIβ antibody were used for immunoprecipitation followed by anti-Mos immunoblotting. IgG, immunoglobulin G. (B) Myc-tagged CKIIβ, $CKII\beta_{1\text{-}160}$, and $CKII\beta_{18\text{-}215}$ RNAs were microinjected into *Xenopus* oocytes. After 3 h, the injected oocytes were treated with progesterone (5 μg/ml) for 12 h. Mos was then immunoprecipitated from these oocytes and analyzed by anti-Myc immunoblotting for the association of Myc-tagged CKIIβ, $CKII\beta_{1\text{-}160}$, and $CKII\beta_{18\text{-}215}$ with Mos. Sizes are indicated in kilodaltons.

peptide antigen competitor (Fig. 4B, even-numbered lanes). GST-CKIIβ was weakly phosphorylated by a nonspecific kinase present in the immunoprecipitates (Fig. 4B, lanes 3 and 4). GST-MKK was constitutively phosphorylated at a low level due to autophosphorylation (Fig. 4B, lanes 5 and 7). Increased phosphorylation of GST-MKK was evident in the presence of v-Mos (Fig. 4B, lane 6). GST-CKIIβ inhibited phosphorylation of GST-MKK by about 40% (after subtraction of the basal level of GST-MKK autophosphorylation) (Fig. 4B, lane 8). The activity of MKK was strongly inhibited. Phosphorylation of $rERK2^{K52R}$ by MKK was reduced by 80% in the presence of CKIIβ (Fig. 4B, lane 8). We conclude that CKIIβ can directly inhibit Mos activity in vitro.

CKIIβ inhibits progesterone-induced *Xenopus* oocyte maturation and MAPK activation. The inhibitory effect of CKIIβ on Mos activity in vitro raised the possibility that it might affect Mos-dependent oocyte maturation. We first examined whether endogenous CKIIβ binds to Mos that is synthesized during *Xenopus* oocyte maturation. CKIIβ was immunoprecipitated from stage VI oocytes, which contain little Mos, or unfertilized eggs, which contain large amounts of Mos. As shown in Fig. 5A, a protein that is the similar size to Mos and reacted with Mos antibody coprecipitated with CKIIβ from eggs but not oocytes. In addition, this protein was not immunoprecipitated by the preimmune serum from either oocyte or egg extracts (Fig. 5A). This finding suggests that the endogenous CKIIβ complexes with Mos in unfertilized eggs. On the other hand, we were not able to detect CKIIβ in Mos immunoprecipitates, possibly because of the insensitivity of CKIIβ antibody for immunoblotting.

To further study the effect of ectopically expressed CKIIβ on progesterone pathway, Myc-CKIIβ, Myc-$CKII\beta_{18\text{-}215}$, and Myc-$CKII\beta_{1\text{-}160}$ RNAs were prepared in vitro and microinjected into oocytes. The injected oocytes were then treated with progesterone to allow endogenous Mos protein synthesis. $CKII\beta_{1\text{-}160}$ was used as a control since it fails to interact with Mos. The expression of Myc-CKIIβ mutants did not interfere with Mos protein synthesis in oocytes treated with progesterone (9). In addition, all of the Myc-tagged proteins, including Myc-$CKII\beta_{1\text{-}160}$, were expressed in the RNA-injected oocytes (Fig. 5B). Moreover, Myc-CKIIβ and Myc-$CKII\beta_{18\text{-}215}$ were detected in Mos immunoprecipitates (Fig. 5B), suggesting that CKIIβ binds via its C terminus to Mos in oocytes as it does in vitro and in yeast.

Injection of Myc-CKIIβ mRNA raised the total CKIIβ protein content by no more than 50% (9), but we found that this moderate increase in CKIIβ expression reduced the sensitivity of oocytes to progesterone. Approximately eight times more progesterone was needed to induce 50% GVBD in CKIIβ-injected oocytes compared with the control oocytes (Fig. 6A). A higher concentration of progesterone (5 μg/ml) could overcome this effect. Control oocytes injected with Myc-$CKII\beta_{1\text{-}160}$ RNA behaved similarly to oocytes injected with water. The inhibitory effect of CKIIβ on GVBD was dose dependent, because GVBD was blocked in two-thirds of oocytes when 30 ng of Myc-CKIIβ RNA was microinjected (Fig. 6B).

We tested whether increased expression of CKIIβ affected CKII holoenzyme activity. CKII kinase activity was increased 1.4-fold in CKIIβ-injected oocytes compared with control oocytes (Fig. 6C). However, a 1.7-fold increase in CKII activity was also observed when control oocytes underwent meiotic maturation, as observed previously (11). Therefore, it seems unlikely that the moderate increase in CKII activity in oocytes expressing Myc-CKIIβ inhibits meiosis. Rather, the inhibition of GVBD by CKIIβ correlated with inhibition of MAPK phosphorylation (Fig. 6D). As MAPK activity is essential for GVBD (33), it is likely that the elevated expression of CKIIβ inhibits progesterone-induced GVBD by repressing Mos-mediated MAPK activation.

CKIIα can rescue the inhibition of oocyte maturation by CKIIβ. Since both Mos and CKIIα bind to the C terminus of CKIIβ (35, 43), we reasoned that if the inhibitory effect of CKIIβ overexpression on oocyte maturation was due to its binding to Mos, overexpression of CKIIα might sequester the excess CKIIβ and rescue the phenotype. To test this hypothesis, Myc-tagged CKIIα mRNA was microinjected into *Xenopus* oocytes. As shown in Fig. 7, either 2 or 20 ng of CKIIα RNAs had little effect on GVBD. By contrast, GVBD was inhibited when oocytes were injected with 20 ng of CKIIβ RNA. When equal amounts of CKIIα RNA and CKIIβ RNAs were coinjected, the percentage of GVBD was increased from 38 to 84%. Taken together, these results lead us to believe that the inhibition of progesterone-induced GVBD by ectopic CKIIβ is likely due to the binding of CKIIβ to Mos, preventing Mos from activating MKK/MAPK, and that ectopic expression of CKIIα can overcome this effect.

DISCUSSION

We have found that CKIIβ binds specifically to Mos in yeast, in vitro, in tissue culture cells and in *Xenopus* oocytes. The C terminus of CKIIβ is necessary and sufficient for this interaction. The binding of CKIIβ to Mos is inhibitory, as CKIIβ represses v-Mos kinase activity and interferes with Mos-mediated MAPK activation in rabbit reticulocyte lysates. When CKIIβ protein is ectopically expressed in *Xenopus* oocytes, it

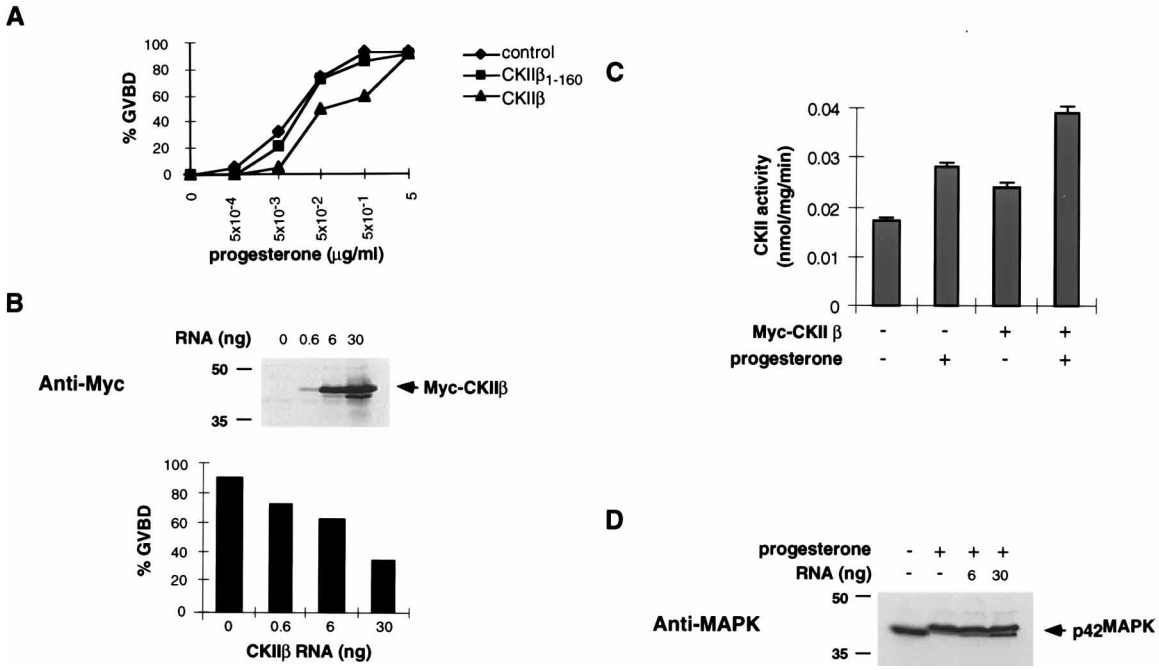

FIG. 6. Effects of CKIIβ on progesterone-induced *Xenopus* oocyte maturation. (A) Oocytes were microinjected with 10 ng of Myc-CKIIβ RNA or 10 ng of Myc-CKIIβ$_{1-160}$ RNA. Three hours later, different concentrations of progesterone were added. Control oocytes were injected with H_2O. Oocytes were then incubated at 16°C for 12 h, and GVBD was scored. The percentages of oocytes undergoing GVBD were calculated (n = 30 to 35 oocytes for each condition). (B) Increasing amounts of Myc-CKIIβ RNA were microinjected into oocytes. Progesterone was added to these oocytes at a final concentration of 0.5 μg/ml. The number of oocytes undergoing GVBD was scored. The expression of Myc-CKIIβ was assayed by immunoblotting. (C) CKII kinase activity was assayed in control oocytes or oocytes injected with 30 ng of CKIIβ RNA in the presence or absence of progesterone. A peptide substrate was used for CKII kinase assay. The incorporation of [γ^{32}-P]ATP into the peptide substrate was measured and converted to the specific activity of CKII. The assay was performed in duplicate. (D) *Xenopus* MAPK phosphorylation was analyzed by anti-MAPK immunoblotting in control oocytes as well as in oocytes microinjected with 6 or 30 ng of Myc-CKIIβ RNA. Sizes are indicated in kilodaltons.

inhibits progesterone-induced GVBD and MAPK activation in a dose-dependent manner. This inhibitory effect can be rescued by ectopically expressing another CKIIβ-binding protein, CKIIα. We propose that ectopic expression of CKIIβ interferes with the progesterone pathway by binding to Mos. Moreover, a significant amount of Mos in unfertilized eggs is complexed with endogenous CKIIβ, suggesting that endogenous CKIIβ may regulate Mos during normal oocyte maturation.

CKIIβ is a subunit of CKII, a serine/threonine kinase that can phosphorylate numerous substrates including many signaling proteins and transcription factors (2, 57). The CKII holoenzyme consists of an $\alpha_2\beta_2$ tetramer of α (or α′) catalytic subunits and β regulatory subunits. The β subunit is critical for stabilizing the structure of the holoenzyme, and it contains both an α-binding domain and a β-dimerization domain. β-β dimerization brings two heterodimers (αβ) together to form a tetramer (22). The regulatory role of the β subunit is complex. Upon binding to CKIIβ, CKIIα changes activity and substrate specificity. CKIIβ stimulates CKIIα activity on most protein and peptide substrates. However, it significantly inhibits calmodulin phosphorylation by CKIIα (5).

There are indications that CKIIβ may have functions other than regulating CKIIα activity. CKIIβ is mainly found in the nucleus partially overlapping with the expression of CKIIα (34, 40). However, some nuclear CKIIα appears to bind not to CKIIβ but to the intranuclear components (73). In fission yeast, overexpression of CKIIβ causes multiseptation and severe growth defects with no more than twofold increase in CKII kinase activity. By contrast, overexpression of CKIIα has no effect (63). A separate finding by Teitz et al. indicates that xeroderma pigmentosum cells gain UV resistance when transfected with CKIIβ cDNA (74). The UV resistance conferred by overexpressing the β subunit may be due to another function of CKIIβ.

Our data suggest that the C-terminal region of CKIIβ is important for Mos binding. The deletion of 55 amino acids from the C terminus of CKIIβ abolishes Mos-binding activity in yeast and in *Xenopus* oocytes. This deletion also abolishes the ability of CKIIβ to associate with CKIIα in a yeast two-hybrid test, in agreement with the published data that deletion of the C-terminal 44 amino acids eliminates β binding to CKIIα (7, 8). By contrast, the N-terminal deletion mutant of β ($\beta_{141-215}$) still associates with Mos. It is possible that the C terminus of β recognizes the common serine/threonine kinase structures between Mos and CKIIα. On the other hand, CKIIβ does not associate with either c-Raf or MKK1, suggesting the specificity of this protein-protein interaction.

As a proto-oncogene product, Mos is regulated at multiple levels. c-*mos* is transcriptionally repressed in somatic cells (61). Ectopic expression of *mos* at a low level transforms somatic cells, and a high level of expression is cytotoxic (52, 54). In germ cells, Mos expression is controlled at the translational level. c-*mos* mRNA is present in stage VI *Xenopus* oocytes but is inefficiently translated (65, 68). When progesterone secreted by the follicle cells causes oocytes to mature, one of the first steps is the cytoplasmic polyadenylation of stored maternal *mos* mRNA to induce Mos protein synthesis (68, 69). It has been shown that once Mos is synthesized, phosphorylation is important for Mos protein stability, its ability to bind to MKK, and its activation (10, 16, 51, 81). The identification of CKIIβ association with Mos suggests another level of Mos regulation. The binding of CKIIβ to Mos inhibits Mos-mediated MKK/

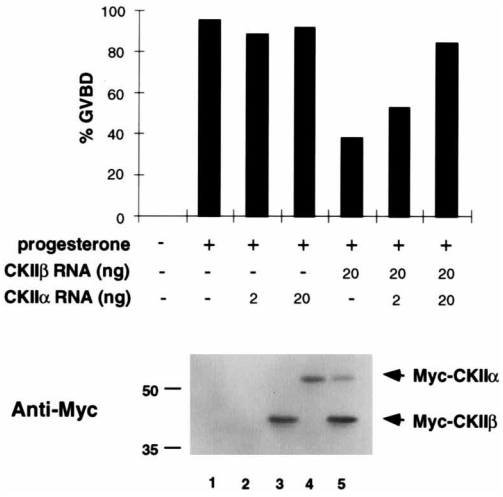

FIG. 7. Rescue of the CKIIβ overexpression phenotype by CKIIα. *Xenopus* oocytes were microinjected with Myc-CKIIβ RNA, Myc-CKIIα RNA, or the combination of two RNAs. Oocytes were incubated in 1× MBS for 4 h, and progesterone was added at 0.5 μg/ml. GVBD was scored 12 h later. Expression of injected mRNAs was assessed by anti-Myc immunoblotting (lower panel). Lanes 1 and 2 represent control oocytes untreated or treated with progesterone. Lanes 3 and 4 represent oocytes injected with 20 ng of Myc-CKIIβ RNA and 20 ng of Myc-CKIIα RNA, respectively. Lane 5 represent oocytes coinjected with 20 ng of Myc-CKIIβ and 20 ng of Myc-CKIIα RNA. Sizes are indicated in kilodaltons.

MAPK activation with partially purified components, in somatic cell lysates and in living *Xenopus* oocytes. The mechanism by which CKIIβ regulates Mos activity remains to be determined, but presumably it involves direct binding since deletion of the Mos-binding domain in CKIIβ prevents regulation of Mos.

Ectopic expression of CKIIβ reduces the sensitivity of *Xenopus* oocytes to progesterone. The extent of inhibition of GVBD depends on the quantity of CKIIβ expressed. The maximum expression of CKIIβ inhibited GVBD by 62%, raised the total CKIIβ content in the oocytes by no more than 50% (9), and increased CKII kinase activity in proportion. A similar increase in CKII kinase activity is detected in CKIIβ-injected oocytes whether or not they undergo GVBD. Indeed, overexpression of CKIIα negates the inhibition by CKIIβ, and the inhibitory effect of ectopic CKIIβ on GVBD correlates with its ability to repress MAPK activation in oocytes. Since progesterone stimulates both MAPK and MPF activities in a Mos-dependent manner, and MAPK activation is essential for progesterone- or Mos-induced oocyte maturation (23, 30, 31, 33, 64, 65), the inhibition of maturation by ectopic CKIIβ likely results from the binding of CKIIβ to Mos and interfering with Mos-mediated MKK/MAPK activation.

The ability of ectopic CKIIβ to inhibit Mos-dependent maturation raises the possibility that endogenous CKIIβ regulates Mos. The protein level of CKIIβ is about the same in oocytes and eggs, and we estimated that a significant amount of Mos is associated with a subset of endogenous CKIIβ in unfertilized eggs (9). How does the endogenous CKIIβ regulate Mos activity? One possibility is that at the initial stage of Mos synthesis, CKIIβ binds to Mos and represses its activity, until the protein level of Mos overcomes a threshold and the free Mos molecules can activate the MAPK pathway (reviewed in reference 18). This is similar to the models for cyclin-dependent protein kinase inhibitor function during the cell cycle (reviewed in reference 50) and may also explain the kinetic difference between MAPK activation in oocytes that are microinjected with Mos protein and oocytes that are treated with progesterone. MAPK is activated within 20 min in oocytes injected with MBP-Mos protein, whereas in the presence of progesterone, MAPK activation does not occur until 2 h after Mos protein synthesis is detected (19, 25, 59, 60). This delay in MAPK activation could be partly explained if newly synthesized Mos is associated with CKIIβ in the progesterone-treated oocytes.

The stoichiometry of endogenous CKIIβ, CKIIα, and Mos in oocytes is not clear. Pulse-chase experiments in several types of cells have shown that the β subunit is synthesized in large excess of α and that only a portion of it forms the tetrameric holoenzyme, with the rest being degraded (41). The excess β subunit may be available to bind to other proteins, such as Mos. Ectopic expression of CKIIα is able to rescue the phenotype of CKIIβ ectopic expression in *Xenopus* oocytes, suggesting that there is a dynamic equilibrium between CKIIα/CKIIβ and Mos/CKIIβ. Both Mos and CKIIβ are localized to the metaphase spindle apparatus in somatic cells (34, 78). Interestingly, CKIIβ is found to be associated with centrosomes where no staining of CKIIα is detected (34). It is possible that Mos and CKIIβ interact at certain subcellular locations and regulate spindle formation through changing MAPK activity. Further studies on the time course of endogenous CKIIβ synthesis, its localization, and its dynamic interactions with Mos and CKIIα in meiosis and mitosis should be useful to reveal the biological significance of CKIIβ binding to Mos.

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