

Regulation of the G₂/M Transition in *Xenopus* Oocytes by the cAMP-dependent Protein Kinase*

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Vertebrate oocytes are arrested in G₂ phase of the cell cycle at the prophase border of meiosis I. Progesterone treatment of *Xenopus* oocytes releases the G₂ block and promotes entry into the M phases of meiosis I and II. Substantial evidence indicates that the release of the G₂ arrest requires a decrease in cAMP and reduced activity of the cAMP-dependent protein kinase (PKAc). It has been reported and we confirm here that microinjection of either wild type or kinase-dead K72R PKAc inhibits progesterone-dependent release of the G₂ arrest with equal potency and that inhibition can be reversed by a second injection of the heat-stable inhibitor of PKAc, PKI. However, a mutant enzyme predicted to be completely kinase-dead from the crystal structure of PKAc, K72H PKAc, was much less inhibitory when carrying additional mutations that block interaction with either type I or type II regulatory subunit. Moreover, inhibition by K72H PKAc was reversed by PKI at a 30-fold lower concentration and with more rapid kinetics compared with wild type PKAc. K72R PKAc was found to have low but detectable activity after incubation in an oocyte extract. These results indicate that inhibition of the progesterone-dependent G₂/M transition in oocytes after microinjection of dead PKAc reflects either low residual activity or binding to regulatory subunits with a resulting net increase in the level of endogenous wild type PKAc. Consistent with this hypothesis, the induction of mitosis in *Xenopus* egg extracts by the addition of cyclin B was blocked by wild type PKAc but not by K72H PKAc. The identification of substrates for PKAc that maintain cell cycle arrest in G₂ remains an important goal for future work.

The oocytes of most species undergo a prolonged arrest in the cell cycle during oocyte growth. In the case of humans, an infant female is born with her lifetime complement of oocytes already arrested in G₂ phase of the first meiotic division. These oocytes can remain quiescent in this arrested state for up to 40 years before initiating the process of oocyte maturation and conversion into an unfertilized egg. It has been evident for many years that this prolonged G₂ arrest of the oocyte is

maintained by a high level of activity of the cAMP-dependent protein kinase. The dependence of G₂ maintenance on the second messenger cAMP was first discovered in amphibian oocytes, which decrease cAMP in response to progesterone to release the G₂ arrest and re-initiate the meiotic cell cycle (1, 2). The enormous size of these oocytes and the resulting ability to microinject them facilitated early experiments that showed that injection of the catalytic subunit of the cAMP-dependent protein kinase (PKAc)¹ blocked the ability of progesterone to initiate oocyte maturation and that microinjection of either the regulatory (R) subunit of PKA (of which there are two termed RI and RII) or the heat-stable inhibitor of PKA, PKI, led to re-initiation of oocyte maturation in the absence of progesterone (3, 4). The demonstration that a decrease in cAMP and PKAc was both necessary and sufficient for meiosis re-initiation was perhaps the first demonstration that all of the effects of a cAMP change could be explained by changes in the activity of PKA, although other targets of cAMP have since been identified (5, 6). It also led to the hypothesis that there must be a substrate of PKA that was an inhibitor of the G₂/M transition in the oocyte (3). The universality of this hypothesis gained support when it was discovered that a cAMP decline shortly after meiosis re-initiation was also evident in the oocytes of both mammalian and invertebrate species (7, 8). The control of cAMP metabolism in the oocytes of various species is regulated differently. In amphibians and fish, progesterone has been proposed to bind to a membrane G protein-coupled progesterone receptor linked to adenylyl cyclase that results in the inhibition of enzyme activity and lower cAMP levels (9–11). However, the nuclear progesterone receptor is also present and has been proposed to mediate maturational events (12, 13). In mammalian oocytes, the cAMP level is thought to be maintained in resting G₂ oocytes either by transfer of cAMP from the surrounding follicle cells into the oocyte (14) or by the activity of a constitutive Gs-linked G-protein coupled receptor that is likely to regulate adenylyl cyclase (15). The disruption of follicle cell/oocyte connections leads to a decline in cAMP in the oocyte and “spontaneous” maturation.

In *Xenopus* oocytes, PKAc was found to inhibit the G₂/M transition, monitored as germinal vesicle (nuclear) envelope breakdown (GVBD), when microinjected before progesterone treatment or even when injected several hours later, shortly before GVBD (3, 16, 17). It has also been shown that PKAc can

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¹ The abbreviations used are: PKAc, catalytic subunit of cAMP-dependent protein kinase; BSA, bovine serum albumin; PKI, heat stable inhibition protein of PKAc; WT, wild type; NEM, *N*-ethyl-maleimide; GVBD, germinal vesicle (nuclear) envelope breakdown; MAPK, mitogen-activated protein kinase; MEK, MAPK/extracellular signal-regulated kinase kinase; MEKK, MEK kinase.

block GVBD induced by microinjection of either recombinant Mos protein, a MEKK that activates the MAPK pathway, or of Cdc25C, the phosphatase that directly dephosphorylates and activates Cdc2 (17, 18). Both the Mos/MAPK pathway and the Cdc25C activation pathway are critical regulators of GVBD in *Xenopus* (for review see Ref. 19). Recently, the inhibition of Cdc25C-induced maturation has been suggested to result from direct phosphorylation of Cdc25C by PKAc to maintain the binding of 14-3-3 proteins, which is believed to prevent the interaction of the phosphatase with its substrate, inactive cyclin B/Cdc2 phosphorylated on Tyr-15 (20).

Whereas phosphorylation of Cdc25C may explain, at least in part, inhibition by PKAc of Cdc25C-induced maturation, other data have suggested that maintenance of G₂ arrest and inhibition of maturation by PKAc may also involve a kinase-independent mechanism. Schmitt and Nebreda (17) observed that an apparent kinase-dead mutant of PKAc (K72R PKAc) was just as effective as wild type (kinase-active) PKAc in inhibiting initiation of progesterone-induced maturation, although K72R PKAc had little effect compared with wild type in blocking GVBD induced by Cdc25C injection. The level of PKA catalytic subunit in the cell is determined by the rates of association and dissociation of regulatory R subunits with PKAc. Therefore, a complication in the interpretation of these types of microinjection experiments is the ability of injected catalytic subunits to associate with the endogenous regulatory subunits, causing a net increase in the level of free wild type oocyte catalytic subunit. *Xenopus* oocytes contain both RI and RII regulatory subunits (17).² Although the interaction with R subunits of several PKAc mutants including the apparent kinase-dead K72R PKAc has been investigated (17), it remains possible that changes in R subunit association or residual kinase activity might account for the ability of K72R PKAc to inhibit GVBD. To investigate this possibility further, we have carried out experiments with PKAc mutants whose interaction with either RI or RII is compromised. The results using a kinase-dead PKAc mutant predicted to be inactive on the basis of the crystal structure indicate that inhibition of GVBD is severely impaired if R subunit interaction is prevented. The data are not consistent with the hypothesis that PKAc inhibits GVBD by a kinase-independent mechanism.

MATERIALS AND METHODS

Generation of Recombinant Proteins—A cDNA encoding the murine PKAc gene (α isoform, a gift from Dr. Susan Taylor, University of California, San Diego, CA) was amplified by PCR and ligated into the bacterial expression vector pET-30 (EK/LIC), which encodes an N-terminal hexa-His tag as part of a 43 amino acid linker upstream of the initiating Met residue of the catalytic subunit. The following PKAc mutants were generated by PCR using Deep Vent DNA polymerase: K72H (kinase-dead); K72R (kinase-dead); R133A (decreased interaction with RII subunit); D328A (decreased interaction with RI subunit); and the double mutants R133A/K72H and D328A/K72H. All of the cDNA constructs were fully sequenced over the entire coding region to confirm mutagenesis and to ensure that no unwanted mutations had been introduced. Recombinant protein was isolated from the bacterial strain BL21(DE3) 20 h after treatment with isopropyl 1-thio- β -D-galactopyranoside at a final concentration of 400 μ M. The His-tagged PKAc mutants were isolated from the soluble bacterial lysate (post-28,000 \times g supernatant) by affinity purification using Talon beads in the presence of 10 mM imidazole and dialyzed into oocyte injection buffer (25 mM Hepes, pH 7.4, 88 mM NaCl, 0.1% β -mercaptoethanol, 0.01% Brij-35). Purified enzyme was stored at -80°C prior to use and diluted to the appropriate concentration in freshly prepared oocyte injection buffer. To generate recombinant PKI, a cDNA encoding rabbit PKI (a kind gift of Dr. Richard Maurer, Oregon Health Sciences University) was amplified by PCR, inserted into the vector pET-30 (EK/LIC), and purified from bacteria as described above. His-tagged PKI was dialyzed into

oocyte injection buffer and stored at -80°C . Protein concentration was determined using the method of Bradford, and protein purity was assessed visually after SDS-PAGE and staining with Coomassie Blue on a 12.5% Anderson gel. Recombinant sea urchin Δ 90 cyclin B used to activate Cdc2 in interphase extracts was expressed in *Escherichia coli* and purified as previously described (21).

Determination of PKAc Enzyme Activity—To determine the specific activity of PKAc and its mutants, 10 ng (5 nM final concentration) of the appropriate recombinant enzyme was assayed in kinase buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 10 mM MgCl₂, 0.1 mM EGTA, 0.1% β -mercaptoethanol, 0.01% Brij-35) using the substrate Kempide (100 μ M) and 100 μ M [γ -³²P]ATP (100 cpm/pmol) for 10 min at 30 $^{\circ}\text{C}$. The reaction was terminated by spotting onto p81-phosphocellulose paper and immersion in 75 mM phosphoric acid. After three additional washes in phosphoric acid, radiolabel incorporation was determined by scintillation counting of the dried paper. To assess the effect of PKI, recombinant His-tagged PKI was included at a final concentration of 5 μ M with preincubation for 10 min on ice prior to initiating the assay at 30 $^{\circ}\text{C}$ as described above. For experiments in cycling egg extracts, histone H1 kinase assays were carried out as described previously (21) and quantified using a PhosphorImager and a Storm 820 scanner (Amersham Biosciences).

Isolation of *Xenopus* Oocytes and Meiotic Maturation—Oocytes were isolated from *Xenopus laevis* ovarian fragments by manual dissection. To induce meiotic maturation, oocytes were incubated in the presence of 10 μ g/ml progesterone. The appearance of a white spot in the center of the animal pole, indicative of GVBD and re-entry into meiosis from the G₂-arrested state, was scored with a dissecting microscope and confirmed by manual dissection after methanol fixation. To assess the effect of the recombinant PKAc proteins on maturation, oocytes were injected with the appropriate recombinant protein (50 nl) for 30 min prior to progesterone addition. Control oocytes were microinjected with BSA (2 mg/ml) or water (50 nl of each) for each experimental series. In the case of double injection experiments (Figs. 3 and 4), the second injection of the appropriate agent diluted in oocyte injection buffer (50 nl) was performed only after control oocytes injected with BSA had re-entered meiosis, as assessed by GVBD. Typically, 50 oocytes were injected per group and all of the experiments were repeated at least twice with similar results. Oocyte lysates were prepared in the presence of okadaic acid (final concentration 3 μ M) 60 min after control oocytes exhibited GVBD. For *ex vivo* comparison of His₆ K72R and K72H PKAc, 1.2 μ g of each enzyme was bound to protein G-Dyna beads (Dyna Corp., Lake Success, NY) coupled to anti-His antibody (Sigma). The beads were incubated in extracts from 100 G₂ oocytes in the presence of 1 mM p-Cl-thio cAMP. After 30 min, beads were re-isolated, washed, and assayed using Kempide as described above with the exception that the assay was done for 30 min at 30 $^{\circ}\text{C}$. Control beads were prepared and assayed identically with the exception that no PKAc was coupled to the beads. Immunoblotting was performed using antibodies that recognize both endogenous and recombinant catalytic PKA α subunits or c-Mos (Santa Cruz Biotechnology) or Cdc2 that is phosphorylated on Tyr-15 (Cell Signaling, Beverly, MA). The equivalent of one oocyte was loaded in each gel lane. Interphase egg extracts were prepared from metaphase egg extracts treated with calcium and cycloheximide as described previously (22).

RESULTS

To carry out these studies, several different mutants of PKAc were cloned, expressed in bacteria as His₆-tagged proteins, and purified to near homogeneity on Talon beads (Fig. 1A). *In vitro* kinase assays confirmed that a kinase-dead variant of PKAc in which the lysine in kinase subdomain II that interacts with the β -phosphates of ATP was mutated to histidine (K72H) had no detectable activity when compared with WT PKAc, whereas a PKAc mutant impaired for interaction with the RII regulatory subunit and PKI (R133A) (23, 24) or the RI regulatory subunit (D328A) (25), had enzymatic activity equivalent to that of the wild type enzyme (Fig. 1A). Initial experiments examined the dose response curve for inhibition of GVBD by wild type or K72H PKAc. As first reported by Schmitt and Nebreda (17) who microinjected mRNA encoding WT and a K72R PKAc mutant, the dose response curve for inhibition was identical for both proteins and complete inhibition was evident when \sim 0.5 pmol of catalytic subunit was introduced (Fig. 1B). Based on these data, for subsequent experiments, oocytes were injected

² P. A. Eyers and J. L. Maller, unpublished data.

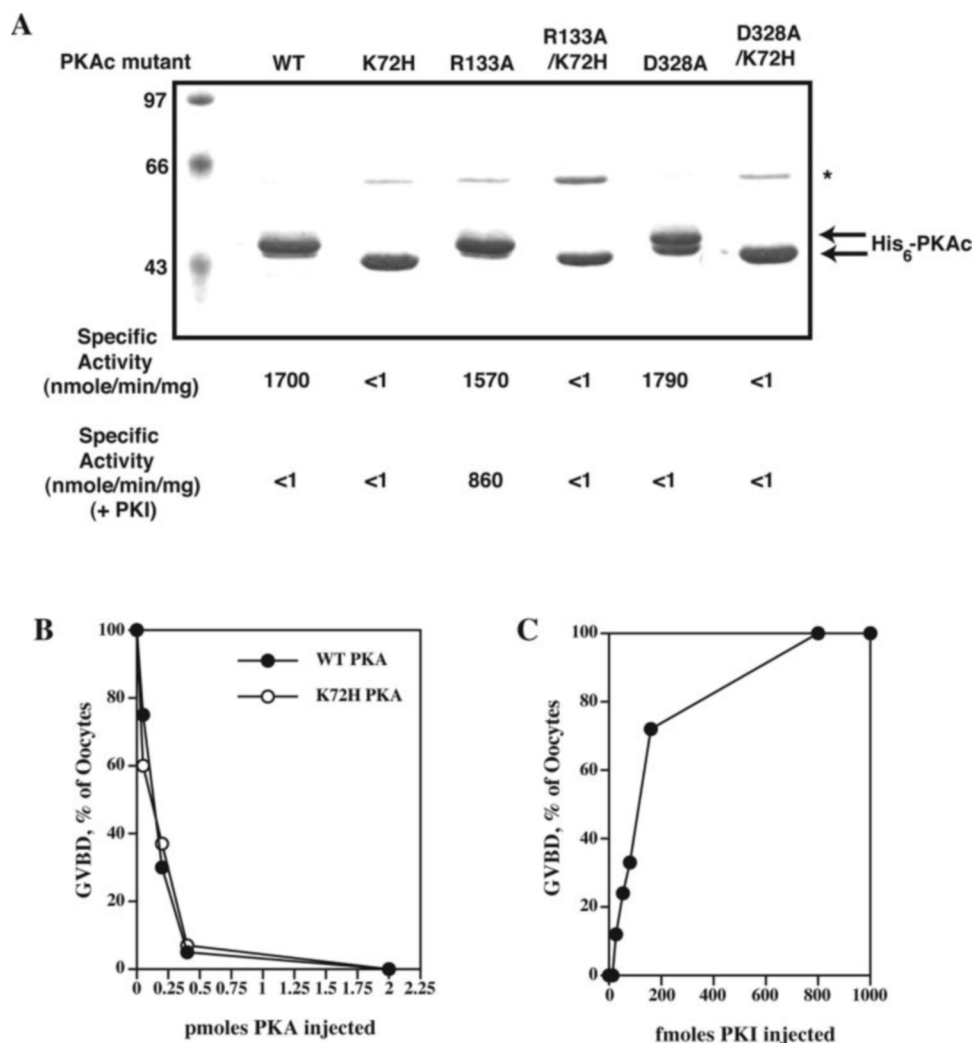


FIG. 1. Effects of PKAc and PKI on oocyte maturation. *A*, biochemical analysis of PKAc. The indicated PKAc proteins were expressed in bacteria and purified as described under "Materials and Methods." The high purity of the preparations is indicated by the presence of only one minor contaminant (asterisk). Differential electrophoretic mobility of the PKA catalytic subunit variants is evident using this gel system (right arrows). The migration of molecular mass markers in kDa is indicated on the left. To determine the specific activity of each protein, 10 ng (5 nM final concentration) of enzyme was assayed as described under "Materials and Methods" in the presence or absence of PKI (5 μ M final concentration, a 1000-fold molar excess). Similar results were obtained in three independent experiments. *B*, dose response curve for inhibition of GVBD by PKAc. Stage VI oocytes were manually dissected from the ovary prior to progesterone treatment as described under "Materials and Methods." Oocytes were microinjected with BSA or the indicated amount of WT PKAc or a kinase-dead variant (K72H), and after 30 min, progesterone was added (final concentration 10 μ g/ml). The appearance of a white spot in the animal pole (signifying GVBD) was scored with a dissecting microscope. Control oocytes were injected with 2 pmol of BSA and typically underwent 90–100% maturation after 5 h of progesterone exposure, identical to water-injected controls. No GVBD occurred in control oocytes that were not exposed to progesterone. Similar results were obtained in several independent experiments using different preparations of PKAc and in oocytes isolated from different frogs. *C*, dose response curve for induction of GVBD by PKI. Stage VI oocytes were dissected as in *A* and microinjected with BSA or the indicated amounts of His₆-PKI. The appearance of a white spot on the animal pole (in the absence of progesterone) was scored with a dissecting microscope. Similar results were obtained in five independent experiments.

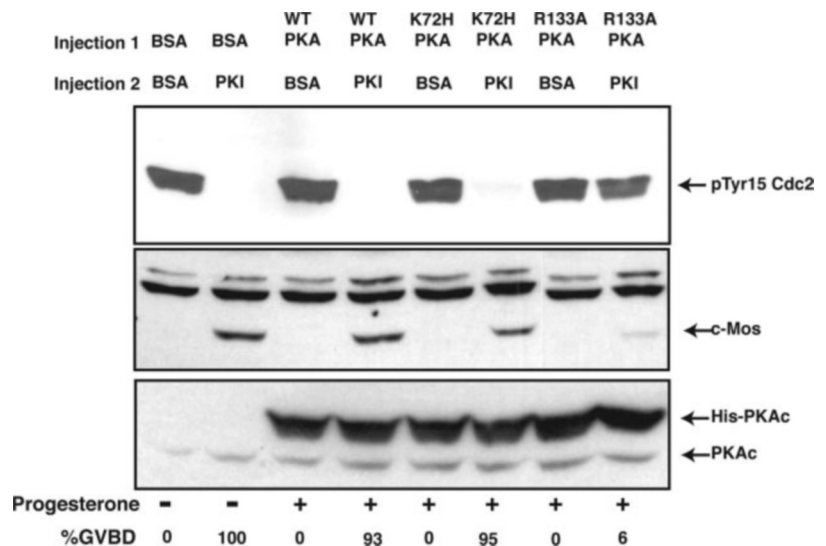
with 1 pmol of various PKAc mutant proteins. We also carried out dose response experiments for the induction of GVBD by PKI and observed that 100% GVBD was obtained with 800–1000 fmol of PKI (Fig. 1C). This dose response curve is consistent with evidence that the concentration of PKA in oocytes is $\sim 1 \mu$ M (3, 17).

Biochemical experiments based on the crystal structure of murine PKAc have established that Arg-133 in PKAc is essential for binding to both RII and PKI (24). Therefore, we compared the ability of purified WT, K72H, and R133A PKAc to inhibit GVBD and associated biochemical effects after microinjection into oocytes. As shown in Fig. 2, WT PKAc completely inhibited progesterone-induced GVBD as well as the Cdc25-dependent dephosphorylation of Tyr-15 in Cdc2. It also blocked the synthesis of Mos, a MEKK responsible for the up-regulation of the MAPK pathway during maturation (for review see Ref.

26). In contrast, microinjection of PKI led to the synthesis of Mos, dephosphorylation of Tyr 15 in Cdc2, and activation of histone H1 kinase activity of which >90% is due to cyclin B/Cdc2 (data not shown) (27). As expected, K72H PKAc inhibited these maturation events in a similar fashion as WT PKAc and inhibition by either type of PKAc was reversed by a subsequent injection of PKI. R133A PKAc, which has activity similar to that of the WT enzyme (Fig. 1), also inhibited GVBD and the associated biochemical changes; however, this was not reversed by a subsequent PKI injection, as predicted from structural and biochemical studies on the interaction of R133A PKAc with PKI and our *in vitro* kinase assays (Fig. 1) (24).

Further experiments examined the kinetics of PKI-induced GVBD and the dose response curve for PKI-induced reversal of inhibition by WT and K72H PKA. As shown in Fig. 3A, after injection of PKI, GVBD occurred much faster in oocytes previ-

FIG. 2. Biochemical analysis of PKAc- and PKI-injected oocytes. Oocytes were initially microinjected with 1 pmol of the indicated control (BSA) or PKAc proteins (*Injection 1*). After GVBD had occurred in 100% of progesterone-treated controls, 8 pmol of the indicated protein (BSA) or PKI (*Injection 2*), was injected as described under "Materials and Methods" and oocytes were incubated until the BSA/PKI-injected oocytes exhibited 100% GVBD. Oocyte lysates were then prepared and immunoblotted with antibodies that recognize either Cdc2 only when phosphorylated on Tyr-15 (*top panel*), c-Mos (*middle panel*) or recombinant (*top arrow*), or endogenous (*bottom arrow*) PKAc subunit (*bottom panel*). The nonspecific band recognized by the c-Mos antibody confirms equal loading of the samples. Similar results were seen in several independent experiments.



ously injected with K72H PKA compared with those injected with wild type PKA, although both PKAc proteins fully blocked GVBD in the presence of progesterone. This is not the result expected if both WT and K72H PKAc use the same non-catalytic mechanism to inhibit GVBD. Similarly, upon comparing the PKI dose response curve for the reversal of inhibition by PKAc, the K72H-injected oocytes were nearly 30 times more sensitive to reversal by PKI than those injected with WT PKAc (Fig. 3B). One explanation for these results is that the K72H PKAc might perturb the re-association of endogenous wild type PKAc with endogenous R subunits, resulting in a net increase in free endogenous WT PKAc activity. Evidence against this possibility is that R133A PKAc is able to inhibit GVBD with an equivalent dose response curve as WT PKAc (Fig. 4B). Although it is clear that R133A does not interact with RII or PKI, it can still interact with RI *in vitro* (17, 25) and Western blotting experiments show that oocytes contain both RI and RII, although their relative levels are not precisely known (data not shown) (17). Biochemical studies based on an analysis of the crystal structure of PKAc have shown that Asp-328 is required for the interaction of the catalytic subunit with RI (25). To evaluate the possible interaction of injected PKAc with RI, we microinjected D328A PKAc into oocytes prior to progesterone treatment. As shown in Fig. 4, D328A PKAc was able to inhibit GVBD and the dose response curve for the inhibition was identical to that of wild type, kinase-dead K72H, and R133A PKAc. Based on these results, it seems possible that interaction with either RI or RII is not required for the inhibition of GVBD by PKAc. However, all of these experiments assessing the importance of R subunit interaction were carried out using PKAc or PKAc mutants that had similar high kinase activity (Fig. 1A). Therefore, we extended the experiment by microinjecting before progesterone treatment 1 pmol (45 ng) of the double mutants R133A/K72H or D328A/K72H PKAc, which similar to the K72H mutant above exhibit no detectable kinase activity compared with WT PKAc in an *in vitro* assay (Fig. 1). The results obtained indicate that these mutants are drastically impaired in their ability to inhibit GVBD (Fig. 4A). To investigate these effects further, we studied the dose dependence of the GVBD block by all of the PKAc proteins. As shown in Fig. 4B, ablation of the kinase activity of either the R133A or D328A mutant of PKAc largely abolished their ability to block GVBD, even when present in the oocyte at levels four times higher than the equivalent PKAc mutants with wild type kinase activity.

The lack of inhibitory activity by the R133A PKAc mutant is in contrast to the report by Schmitt and Nebreda (17) that

R133A PKAc with a K72R substitution still exhibited full inhibitory activity toward progesterone-induced GVBD. One possible explanation is suggested by the report (28) that yeast PKAc with a K116A substitution still exhibited 0.3% wild type activity and could support obligate PKA-dependent growth of the yeast cell. In our studies, we have used PKAc with a K72H substitution, which is predicted to be completely kinase-dead on the basis of the crystal structure and numerous biochemical studies (23). To address this issue directly, we incubated the K72R and K72H PKAc enzymes on Dyna beads in G_2 oocyte extracts in the presence of cAMP to block any effects of R subunit association. After re-isolation and washing, assays were performed with saturating concentrations of Kemptide as substrate. As shown in Fig. 5, the K72R enzyme had significantly more activity than the K72H enzyme, ~8 times above the background with control beads in the assay. The specific activity of the K72R PKAc enzyme was ~2.8 nmol/min/mg or 0.16% activity of WT PKAc (Fig. 1). This level of activity would not have been detectable in the assays comparing WT and K72H PKAc (Fig. 1A), because in those assays, the amount of PKAc in the assay is >40-fold lower to ensure that WT PKAc activity is measured in the linear range of the assay. These results suggest that, even in the absence of R subunit interaction, the K72R enzyme possesses sufficient catalytic activity to mediate G_2 arrest of the oocyte.

The experiments described so far were carried out in living frog oocytes induced to undergo the meiotic cell cycle transitions of maturation. It was important to know whether PKAc activity was also required for control of normal mitotic divisions. Therefore, we sought to extend our study to frog egg extracts executing mitotic cell cycles. Extracts from activated eggs can carry out up to three consecutive cycles of mitosis and DNA synthesis with kinetics similar to that of the intact embryo (21, 29). Previous studies have shown that these cycles are driven by oscillatory cycles of synthesis and degradation of cyclin B; however, superimposed on changes of cyclin B/Cdc2 activity are oscillations in the activity of PKA (21). In particular, cAMP and PKAc activity decline just before entry into mitosis and cAMP and PKAc activity increase at the metaphase/anaphase transition (29). These oscillations are important, because elevated PKAc can block entry into M phase and RII can block exit from metaphase, an effect reversed by cAMP addition (21). In extracts arrested in metaphase by cytostatic factor (for review see Ref. 26), a convenient way to examine requirements for entry into mitosis is to add cycloheximide to prevent cyclin B synthesis after the release of metaphase arrest

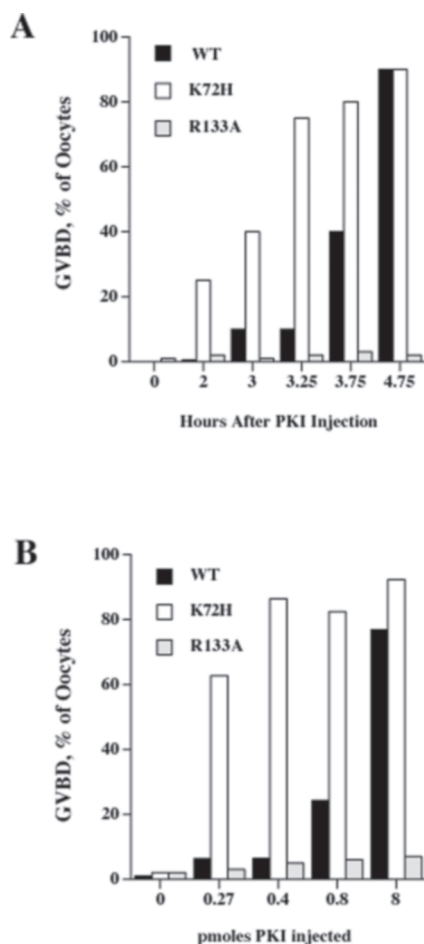


FIG. 3. Reversal of PKAc Inhibition by PKI. *A*, kinetics of GVBD in PKI-injected oocytes. 50 oocytes were injected with 1 pmol of the indicated PKAc protein and, 30 min later, treated with progesterone (10 μ g/ml final concentration). When control (BSA-injected) oocytes had undergone GVBD, each group of PKAc-injected oocytes was injected a second time with 8 pmol of PKI, and at the indicated times, the percent of oocytes that had undergone GVBD was determined by microscopic inspection. Similar results were obtained in two independent experiments. *B*, dose response curve for PKI-induced GVBD. Oocytes were initially treated as in *A*, and when control (BSA-injected) oocytes had reached 100% GVBD, each group of PKAc-injected oocytes was injected a second time with the indicated concentration of PKI. In each group of 50 oocytes, the percentage that had undergone GVBD was scored after control oocytes (injected with 0.27 pmol PKI) exhibited GVBD in the absence of progesterone (*cf.* Fig. 1C). Similar results were obtained in two independent experiments.

by calcium addition, resulting in an interphase extract that is dependent upon the addition of exogenous cyclin B for elevation of cyclin B/Cdc2 histone H1 kinase activity and entry into the next mitosis. As reported previously (29), the addition of a non-degradable form of sea urchin cyclin B, Δ 90 cyclin B, to an interphase extract led to the activation of cyclin B/Cdc2 histone H1 kinase activity and M phase entry by 90 min (Fig. 6, *control*). However, M phase entry was blocked by the addition of cAMP or WT PKAc but not by K72H PKAc (Fig. 6A). The effect of WT PKAc was reversed by the addition of PKI. The resulting histone H1 kinase activity was even higher than control, presumably because of inhibition of both exogenously added and endogenous PKAc by PKI. The inhibition of Cdc2 activation was accompanied by elevated levels of inhibitory phosphorylation of Tyr-15 in Cdc2 (Fig. 6B). These results indicate that, in addition to its requirement for regulating the meiotic G₂/M transition in oocytes, the catalytic activity of PKAc is also necessary to inhibit cyclin B-dependent mitotic entry in *Xenopus* egg extracts.

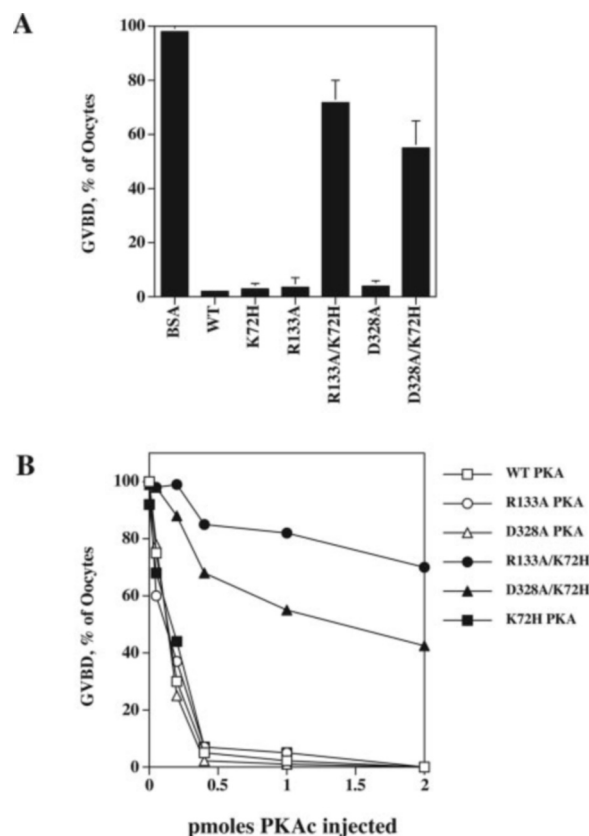


FIG. 4. Effect of regulatory subunit interaction on inhibition of GVBD by PKAc. *A*, inhibition of GVBD requires R subunit interaction. Oocytes were prepared as in the legend to Fig. 1 and injected with 1 pmol of the indicated PKAc mutant or BSA. After 30 min, progesterone (10 μ g/ml final concentration) was added to initiate meiotic re-entry. After 8 h, the percentage of oocytes that had undergone GVBD was calculated. The data shown are the mean \pm S.E. of three independent experiments. *B*, dose response of PKAc mutants. The same experiment was performed as in *panel A* with the exception that oocytes were injected with the indicated amounts of each PKAc protein 30 min prior to progesterone exposure. GVBD was assessed in groups of 50 oocytes for each PKAc protein. Identical results were obtained in two independent experiments.

DISCUSSION

The results presented in this paper are of interest in relation to the hypothesis that the inhibition of oocyte maturation and thus maintenance of G₂ arrest by PKAc occurs by a kinase-independent mechanism (17). Our work confirms the data from this previous report (17), which demonstrates that R133A PKAc (impaired in binding to RII and PKI) is equally potent at inhibiting GVBD as wild type or K72H PKAc. This was originally interpreted to mean that the effects of injected PKAc could not be a result of interaction with the endogenous RII subunit, which would lead to a net increase in free endogenous PKAc subunit. However, the data presented here suggest that both the RI and RII present in oocytes must interact with kinase-dead K72H PKAc because K72H mutants that are unable to bind either RI or RII are dramatically reduced in their ability to inhibit GVBD. Similarly, the amount of PKI required to reverse inhibition by K72H PKAc is much lower than that for WT PKAc, even though both are equally capable of blocking GVBD (Fig. 3), and the amount of PKI needed to reverse the inhibition of GVBD is much lower for K72R PKAc-injected oocytes than for those injected with WT PKAc (Fig. 3B). This result is most consistent with the recombination of the injected K72H PKAc with endogenous RI or RII, causing an increase in the level of endogenous free catalytic subunit in the oocyte. Less PKI would be required to induce GVBD after K72H PKAc

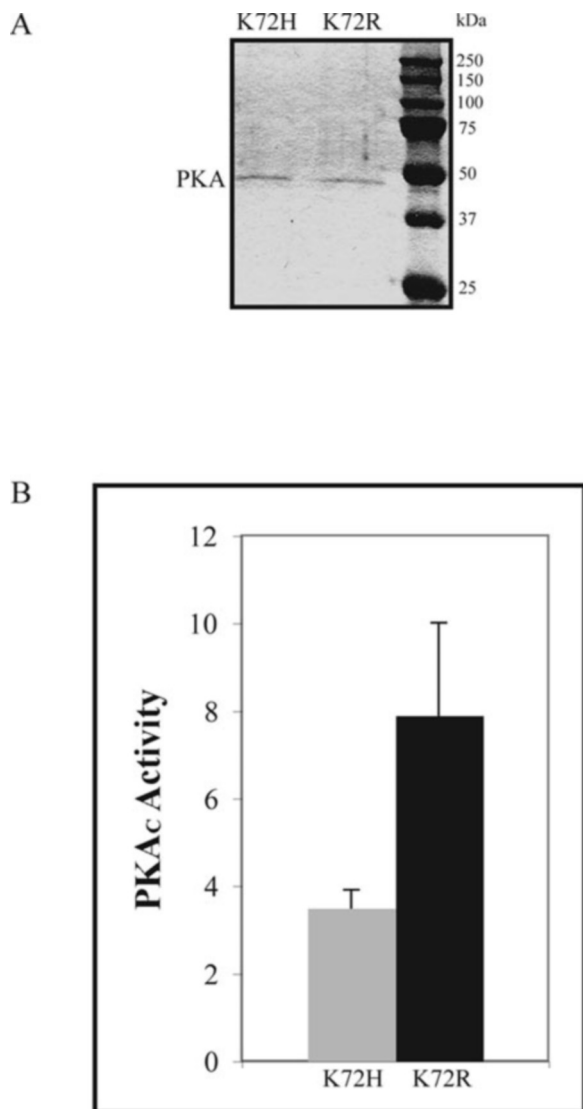


FIG. 5. *Ex vivo* activity of K72R and K72H PKAc. As described under "Materials and Methods," 1.2 μ g of either His₆-K72H or His₆-K72R PKAc were bound to protein-G Dynal beads and incubated for 30 min at 22 °C in extracts from G₂-arrested oocytes in the presence of cAMP to prevent R subunit interaction. Control beads were incubated identically with the exception that no PKAc was bound. After washing, assays were performed as described under "Materials and Methods." A, Coomassie Blue-stained gel of the PKAc enzymes assayed in panel B. B, the results of kinase assays are shown for three independent experiments as relative fold activity over control beads. The specific activity of K72R PKAc was calculated as 2.77 nmol/min/mg or ~0.16% activity of WT PKAc (Fig. 1). The results are expressed as the mean \pm S.E. ($n = 3$).

is injected, because the only catalytically active PKAc in the system would be the endogenous oocyte enzyme. In the case of R133A PKAc, this enzyme could still re-associate with RI and thus also cause a net increase in the level of oocyte free PKAc. Regardless of their interaction with the regulatory subunits, both R133A and D328A PKAc exhibit wild type kinase activity, which is presumably sufficient to maintain meiotic G₂ arrest, even when R subunit interaction is blocked. Importantly, however, K72H PKAc loses considerable effectiveness at inhibiting GVBD when interaction with either R subunit is blocked. The residual inhibition of GVBD by either double mutant might still reflect changes in endogenous PKAc because of re-association of injected PKAc with the other R subunit. The level of impaired inhibition was greater for R133A/K72H PKAc (Fig. 5B), perhaps reflecting a greater abundance of RII over RI, as

suggested by early biochemical studies (30).

The reduced inhibitory activity of PKAc with a mutation that impairs interaction of PKAc with both RI and RII (W196R) (31) was previously reported by Schmitt and Nebreda (17) and is consistent with the data presented in Fig. 4 for PKAc carrying a mutation at Arg-133 or Asp-328. However, Schmitt and Nebreda (17) reported that R133A K72R PKAc potentially inhibited GVBD. The basis for this discrepancy is unclear at present, but Schmitt and Nebreda (17) injected mRNA for PKAc, whereas we have injected bacterially synthesized purified PKAc proteins of known concentration and activity. We have also carried out a dose response curve for inhibition, which demonstrates that, even at levels four times greater than wild type, R133A K72H PKAc is unable to substantially inhibit progesterone-induced GVBD (Fig. 4B). Another difference between the studies is the kinase-dead mutant employed. Schmitt and Nebreda (17) used K72R PKAc in their experiments, whereas we used K72H PKAc. K72H PKAc is predicted to be completely inactive on the basis of the crystal structure of PKAc (23). Significantly, in an oocyte extract, K72R PKAc had greater residual kinase activity than K72H PKAc (Fig. 5B), equivalent to 0.16% wild type activity, perhaps because the substitution of Arg for Lys results in a similar positively charged residue at the ATP-binding interface. Indeed, budding yeast PKAc with an even less conservative mutation (K116A) has been reported to possess activity equivalent to 0.3% wild type enzyme (28), sufficient to support cell growth, and K297R p60^{C-Src} exhibits activity equal to 0.5% wild type enzyme.³ Other protein kinases, such as Aurora A, retain as much as 10% wild type activity with the equivalent Lys to Arg mutation when assayed carefully with physiological substrates and are fully phosphorylated on the T-loop (32, 33). Because of the problematic nature of the effect on the activity of amino acid substitutions for the active site lysine of protein kinases, we suggest that mutating the Asp residue in the Asp-Phe-Gly motif that affects Mg²⁺ binding is more certain to result in a completely dead enzyme (32, 33).

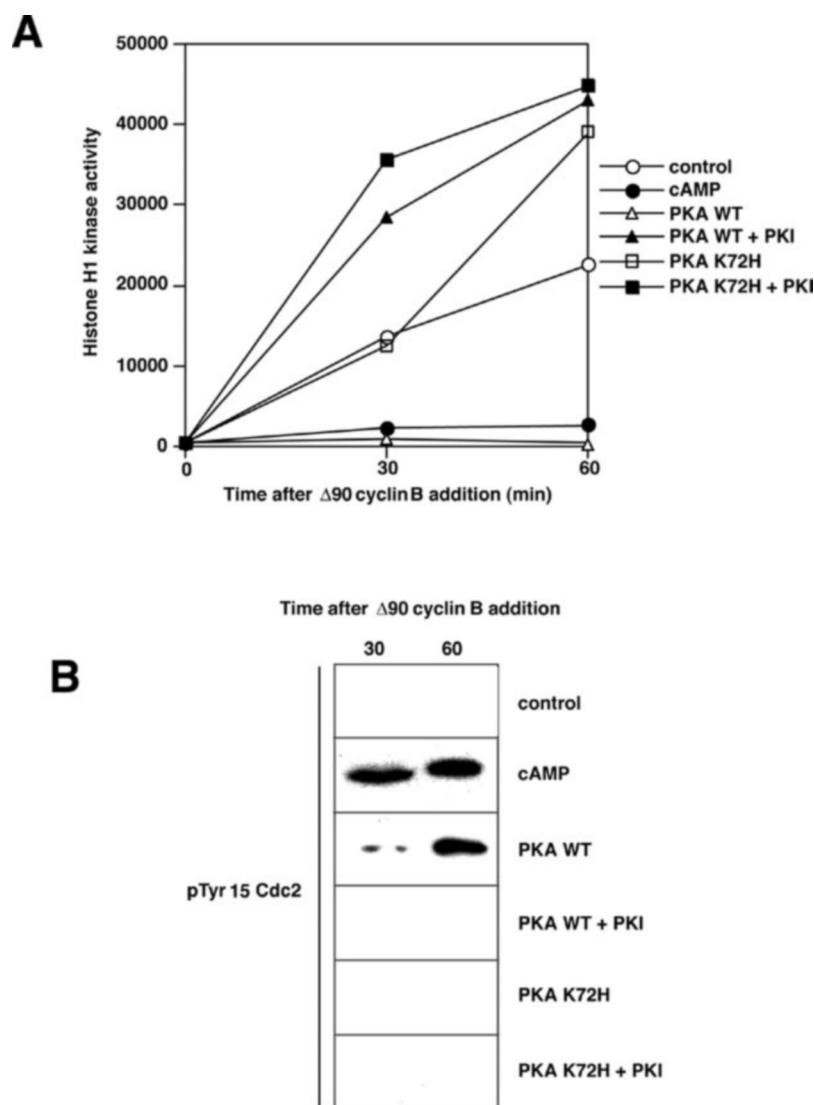
The original report over a quarter of a century ago by Maller and Krebs (3) showing that microinjection of PKAc inhibited progesterone-induced GVBD was conducted before the advent of molecular cloning. The control protein in those experiments was PKAc that had been inactivated by treatment with the cysteine-alkylating agent, *N*-ethyl-maleimide (NEM), which abolished detectable protein kinase activity (3). It is interesting to note that recent studies on NEM treatment of PKAc show that Cys-199 is the preferred target for NEM and that alkylation at this site impairs not only catalytic activity but also the ability of PKAc to interact with both R subunits.⁴ In light of the results presented here, it may be that the failure to inhibit GVBD by NEM-treated PKAc was due to the failure to interact with R subunits, rather than to the loss of catalytic activity by alkylation.

If, as we suggest, catalytic activity of PKAc is responsible for blocking even early initiating events of maturation, an important question concerns what substrate is being phosphorylated to maintain the G₂ block of the oocyte. Identification of a high affinity, physiological substrate might permit a rigorous assessment of endogenous PKAc activity. Overexpression of a non-physiological PKAc substrate in oocytes has confirmed predictions of a decrease in PKAc activity with progesterone treatment (34); however, kinase assays with crude extracts and similar nonspecific substrates have not revealed a detectable increase in total extract PKAc activity after expression of K72R

³ T. Hunter, personal communication.

⁴ S. Taylor, personal communication.

FIG. 6. Inhibitory effect of PKAc on $\Delta 90$ cyclin B-mediated activation of Cdc2. A, PKAc inhibits stimulation of histone H1 kinase activity by cyclin B. Interphase egg extracts were prepared from unfertilized *Xenopus* eggs as described under "Materials and Methods." $\Delta 90$ cyclin B was added 60 min after the addition of calcium (0.4 mM) and cycloheximide (10 μ g/ml) to a cytostatic factor (metaphase-arrested) extract, resulting in exit from M phase and arrest in interphase after degradation of cyclin B. The following were added at the same time as $\Delta 90$ cyclin B: buffer (open circles); 1 μ M cAMP (filled circles); 1 μ M wild type recombinant PKAc protein (PKA WT, open triangles), 1 μ M wild type recombinant PKAc protein and 20 μ M PKI (PKA WT + PKI, filled triangles), 1 μ M kinase-dead PKAc (PKA K72H, open squares); or 1 μ M kinase-dead PKAc and 20 μ M PKI (PKA K72H + PKI, closed squares). Cyclin B/Cdc2 activation was assessed by measuring histone H1 activity following the addition of $\Delta 90$ cyclin B. B, inhibitory Tyr-15 phosphorylation of cyclin B/Cdc2. The extracts in panel A were immunoblotted at the indicated times for pTyr-15 in Cdc2 using a phosphospecific antibody as described under "Materials and Methods."



PKAc (17). This negative result with a nonspecific substrate should be interpreted cautiously. For example, Aurora A with a non-phosphorylatable amino acid in the T-loop (T295V Aurora A) has no detectable activity against its usual nonspecific substrate, histone H3, but it has detectable activity against a high affinity substrate, TPX2 (33).

A possible candidate PKAc substrate would be the Cdc25C phosphatase, which is responsible for direct dephosphorylation and activation of cyclin B/Cdc2. Recently, Ruderman and colleagues (20) demonstrated that phosphorylation by PKAc generates a 14-3-3 protein-binding site that impairs the interaction of Cdc25C with its substrate, pTyr-15 Cdc2/Cyclin B. The failure of Nebreda and co-workers (17) to inhibit GVBD caused by Cdc25C injection with K72R PKAc suggests that this reaction is critical and may explain at least in part the necessity for full catalytic activity to inhibit Cdc25C-induced GVBD. It is possible that this phosphorylation could also be involved in the requirement of PKAc activity to block M phase upon cyclin B addition (Fig. 6), because the level of Tyr-15 phosphorylation of Cdc2 is increased by the addition of wild type PKAc. However, in G_2 phase oocytes, we have been unable to detect an increase in Cdc25C phosphorylation after the injection of K72H PKAc (data not shown). Another possibility that merits further investigation is regulation by PKAc of a kinase that is able to phosphorylate Tyr-15 in Cdc2. It is evident that very little endogenous PKAc activity is needed to maintain G_2 arrest,

because very low concentrations of PKI rapidly reverse inhibition by K72H PKAc (Fig. 3). On the other hand, it seems clear that full catalytic activity of PKAc is needed to block GVBD induced by Cdc25C injection because K72R PKAc is unable to effectively block GVBD compared with WT PKAc (17). It is not uncommon for different levels of activity of a protein kinase to be required for different *in vivo* reactions, which may take place at different cellular locations. For example, partial inhibition of the polo-like kinase Plx1 by antibody injection has only a modest effect on its requirement for activation of Cdc25C in prophase but completely disrupts the ability of Plx1 to organize the bipolar spindle at metaphase (35).

In any case, it is evident that additional effects of PKAc must also be involved in maintaining cell cycle arrest. For example, GVBD induced by PKI injection (Fig. 1B) still requires protein synthesis (16, 17, 19). In addition, in egg extracts, the activity of cyclin B/T14AY15F Cdc2, which is not a substrate for Cdc25C, is still inhibited upon the addition of PKA (data not shown).⁵ These results suggest that other substrates for PKAc besides Cdc25C may affect protein synthesis or other signaling pathways that contribute to maintenance of the G_2 arrest of the oocyte.

⁵ N. R. Hayashi and J. Gautier, unpublished data.

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