Vol. 182, No. 1, 1992 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 15, 1992 Pages 105-114

CHARACTERIZATION OF PROTEIN KINASE C IN *XENOPUS* OOCYTES

Setsuko Sahara[1], Ken-ichi Sato[1], Mamoru Aoto[1], Tetsuo Ohnishi[1], Hiroshi Kaise[1], Hiroshi Koide[2], Kouji Ogita[2], and Yasuo Fukami[1]*

[1]Laboratory of Molecular Biology, Biosignal Research Center, Kobe University, Kobe 657, Japan

[2]Department of Biochemistry, Kobe University School of Medicine, Kobe 650, Japan

Received October 31, 1991

SUMMARY: Protein kinase C (PKC) was partially purified from *Xenopus laevis* oocytes by ammonium sulfate fractionation followed by DEAE-cellulose and hydroxyapatite column chromatography. In the latter chromatography, two distinct PKC activities were identified. Both PKC fractions contained an 80 kDa protein which was recognized by three antisera raised against the conserved regions of mammalian PKC. However, specific antisera against α, βI, βII, and γ-subspecies of rat PKC did not recognize the protein. Kinetic properties of the *Xenopus* PKCs were very similar to those of the rat αPKC, and only a subtle difference was found in the mode of activation by arachidonic acid. When oocytes were treated with the tumor promoter, phorbol 12-myristate 13-acetate, one of the *Xenopus* PKCs was found to disappear very rapidly, while the other remained unchanged up to 2 hr.

Protein kinase C (PKC) is a large family of proteins with multiple subspecies (1) and plays crucial roles in signal transduction (2). In *Xenopus* oocytes, involvement of PKC in their maturation and fertilization has been suggested by following observations: a) phorbol 12-myristate 13-acetate (PMA) induces fertilization-like events such as cortical granule exocytosis, cortical contraction, and cleavage furrow formation (3), b) PMA induces germinal vesicle breakdown (GVBD) (4), which is a morphological indication of the process termed oocyte maturation (5), c) microinjection

* To whom correspondence should be addressed.

0006-291X/92 $1.50

of rat brain PKC accelerates insulin-induced but not progesterone-induced oocyte maturation (4, 6), d) injection of PKC potentiates the oocyte maturation and ribosomal S6 protein phosphorylation induced by oncogenic *ras* protein (7), and e) a mutant construct of PKC that is constitutively active in the absence of PMA can induce GVBD, when expressed in oocytes (8). To clarify potential roles of PKC in *Xenopus* oocytes, it is essential to identify and characterize this enzyme in these cells. Although two cDNA clones which resemble those encoding mammalian PKCs have been isolated (9), enzymological properties of *Xenopus* PKC (xPKC) have not been well studied. The present paper will describe the purification and characterization of xPKC.

MATERIALS AND METHODS

Materials: Adult *Xenopus laevis* were obtained from Hamamatsu Biomaterials (Hamamatsu, Japan). The *lon* protease-deficient strain of *Escherichia coli*, AD18, was kindly provided by T. Yura (Kyoto University), and cultured in kanamycin-supplemented LB medium (10). Calf thymus H1 histone was prepared as described previously (11). [γ-^{32}P]ATP (35020) was obtained from ICN.

Antibodies: Various antibodies were prepared to identify the conserved and variable regions of PKC subspecies. Mammalian PKCs have a common structure consisting of five variable (V1 to V5) and four conserved regions (C1 to C4) (reviewed in ref. 1). Antisera against C1 and C2 regions of the γ-subspecies of rat PKC (γPKC) were obtained by immunizing rabbits with the polypeptides corresponding to these regions which were expressed as an inclusion body in *E. coli* transfected with the plasmids pTB967 and pTB968 (12), respectively. Antiserum against the kinase domain (C3+C4) of γPKC was prepared in a similar manner except that *E. coli* AD18 was used as a host for expression of the plasmid pUCγKD, in which a BamHI-EcoRI fragment (corresponding to the kinase domain) of γPKC cDNA (13) was ligated to the pUC19 vector (10). Anti-peptide antisera against the V5 region of the α, βI, βII, and γ-subspecies of rat PKC were prepared as described by Kishimoto *et al.* (14).

Enzyme and Assay: The rat brain α, β, and γ-subspecies of PKC were purified as described (15). PKC was routinely assayed by measuring the incorpration of ^{32}P into H1 histone from [γ-^{32}P]ATP under the conditions described previously (16). The standard reaction mixture (50 μl) contained 20 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 10 μM [γ-^{32}P]ATP (3,000 cpm/pmol), 8 μg/ml of phosphatidylserine (PS), 0.8 μg/ml of diolein (DO),

0.1 mM $CaCl_2$, and 0.2 mg/ml of H1 histone. The basal activity was measured in the presence of 0.1 mM EGTA instead of $CaCl_2$, PS and DO. After incubation for 5 min at 30°C, the reaction was terminated by spotting a 40 µl aliquot of the reaction mixture onto P81 phosphocellulose paper (Whatman). The paper was washed five times with 75 mM H_3PO_4, and the radioactivity was determined by Cerenkov counting.

Preparation of Oocytes: Oocytes were manually dissected from the ovary and treated with 0.5% collagenase in OR-2 buffer (17) [5 mM HEPES (pH 7.5), 82.5 mM NaCl, 2.5 mM KCl, 1 mM Na_2HPO_4, 1 mM $MgCl_2$, and 1 mM $CaCl_2$] for 2 hr at room temperature. The defolliculated oocytes were washed five times with OR-2 and then once with ice-cold homogenizing buffer [20 mM Tris-HCl (pH 7.5), 10 mM EGTA, 2 mM EDTA, 10 mM β-mercaptoethanol, 0.1 mg/ml of leupeptin, and 50 µM (*p*-amidinophenyl)-methanesulfonyl fluoride hydrochloride (APMSF)] prior to the homogenization.

Isolation of xPKC: All manipulations were carried out at 0-4°C. Oocytes (ca. 60 ml in a packed volume) suspended with 60 ml of homogenizing buffer were disrupted in a Dounce homogenizer (20-30 strokes). The homogenate was centrifuged for 20 min at 15,000 x g. The crude extract was centrifuged further for 1hr at 100,000 x g. The supernatant and fluffy part of the pellet that contains cytoplasmic vesicles (18) were taken together and diluted with homogenizing buffer to a final volume of 120 ml (2 x volume of the starting oocytes). To this sample, Triton X-100 (final 1%) was added and mixed well. After 30 min, saturated $(NH_4)_2SO_4$ was added to give 33% saturation, and the mixture was standed on ice for 1 hr. Protein was not salted out at this step. However, by centrifugation for 20 min at 15,000 x g, a large amount of lipids was separated over the aqueous phase. The lipid layer was removed manually. After removing the residual lipids by glasswool, the sample was brought to 75% $(NH_4)_2SO_4$ saturation and standed on ice for 30 min. At this step, protein was precipitated and recovered by centrifugation for 20 min at 15,000 x g. The precipitate was dissolved in 5 ml of buffer A [20 mM Tris-HCl (pH 7.5), 0.5 mM EGTA, 0.5 mM EDTA, 10 mM β-mercaptoethanol, 20 µg/ml of leupeptin, and 50 µM APMSF] and dialyzed against buffer A for 8-10 hr. The dialyzed fraction was clarified by centrifugation, and the refractive index of the supernatant was adjusted with buffer A to that equivalent to buffer A containing 20 mM NaCl. The sample was brought again to 1% Triton X-100, standed on ice for 30 min, and then applied to a DEAE-cellulose column (DE52, 3.5 x 3.0 cm) equilibrated with buffer A. The column was washed with 150 ml of buffer A containing 0.5% Triton X-100, then with 300 ml of buffer A containing 20 mM NaCl. The enzyme was eluted stepwise with 90 ml of buffer A containing 150 mM NaCl. The eluate was applied directly to a hydroxyapatite column (Koken, 0.78 x 10 cm) which was connected to an FPLC system (Pharmacia) and equilibrated with buffer B [20 mM potassium phosphate (pH 7.5), 0.5 mM EGTA, 0.5

mM EDTA, 10% glycerol, 10 mM β-mercaptoethanol, 20 μg/ml of leupeptin, and 50 μM APMSF]. The column was washed with 50 ml of buffer B at a flow rate of 0.4 ml/min. The enzyme was eluted by application of a 50-ml linear concentration gradient (20 to 300 mM) of potassium phosphate in buffer B. Fractions (0.5 ml each) were collected and assayed for PKC activity using H1 histone as substrate.

Immunoblot Analysis: Samples were separated by SDS-polyacrylamide gel electrophoresis (19) and transferred to a polyvinylidene difluoride membrane by using a semi-dry blotter (Sartorius). The membrane was soaked in T-TBS [20 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.05% Tween 20] containing 1% skim milk for 1 hr, then incubated with the first antibody diluted in the T-TBS/skim milk solution for 16 hr at 4°C. After washing with T-TBS, the membrane was incubated with alkaline phosphatase-conjugated anti-rabbit IgG (Cappel) for 1 hr at room temperature. To detect the immune complex, 5-blomo-4-chloro-3-indolylphosphate *p*-toluidine salt was used as substrate.

RESULTS AND DISCUSSION

Isolation of Two Types of xPKC: Since oocytes contain a large amount of lipids, it was essential to remove lipids from the extract by ammonium sulfate fractionation as described in MATERIALS AND METHODS. The lipid-dependent activity could be detected only after DEAE-cellulose column chromatography and purified further by hydroxyapatite column chromatography. As shown in Fig. 1A, xPKC was eluted in two distinct peaks with lipid-dependent protein kinase activity. The first peak was designated as xPKC I and the second peak as xPKC II. Coinciding with the activity of both xPKC fractions, a single polypeptide band with 80 kDa was detected by immunoblotting with antiserum against the kinase domain of γPKC (Fig. 1B). Antiserum against the C1 region of γPKC, that is essential for PMA-binding (12), and antiserum against the C2 region, that is responsible for the Ca^{2+}-dependency (12), also recognized the 80 kDa protein in both fractions. However, anti-peptide antisera against the V5 region of α, βI, βII, and γ–subspecies of rat PKC did not react with the 80 kDa protein.

These results indicate that the two xPKCs are closely related to the mammalian PKC in their conserved regions but are distinct from the α, βI, βII and γ–type of PKC. Chen *et al.* (9) isolated and sequenced two *Xenopus* cDNA clones, which have a considerable sequence homology with α and β–

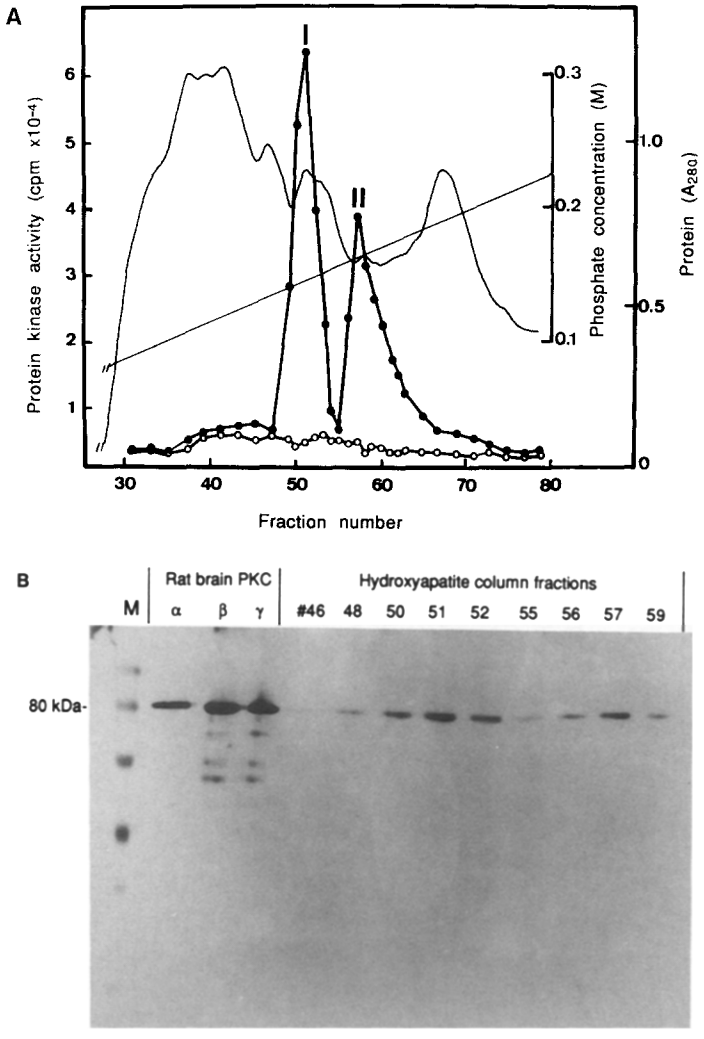

Fig. 1. Hydroxyapatite column chromatography of xPKC. (A) DEAE-cellulose fraction of xPKC was applied to a hydroxyapatite column as described in the text. PKC activity was assayed in the presence of 0.1 mM $CaCl_2$, 8 μg/ml of PS and 0.8 μg/ml of DO (●) or in the presence of 0.1 mM EGTA (○) instead of $CaCl_2$, PS and DO. Protein was monitored by absorbance at 280 nm (——). (B) Immunoblot analysis of xPKC. The hydroxyapatite column fractions (30 μl each) were subjected to SDS-polyacrylamide (12.5%) gel electrophoresis and analyzed by immunoblotting with antiserum against the kinase domain of γPKC. Purified rat brain α, β and γ-subspecies of PKC were included as a positive control. M, Prestained PAGE standards (Bio-Rad).

subspecies of mammalian PKC. Whether these messages are translated into proteins and correspond to the present xPKCs is to be determined. A possibility may not be ruled out for the existence of additional xPKC subspecies in oocytes.

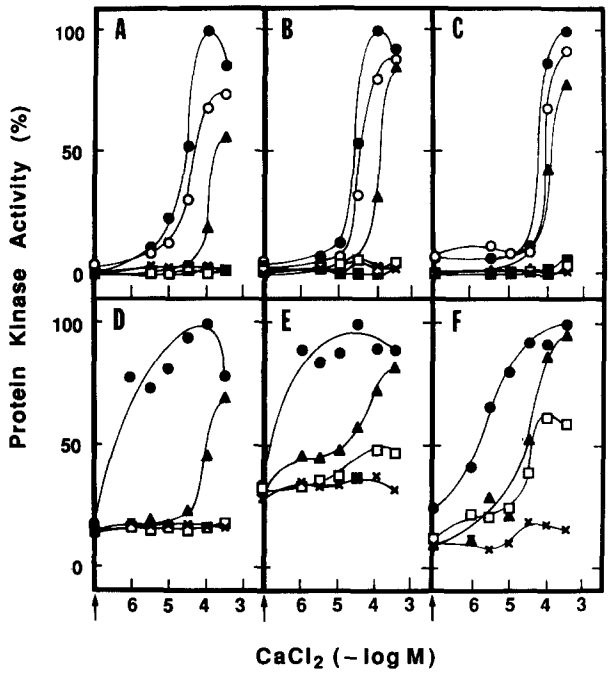

Fig. 2. Activation of PKC by PS, DO and PMA in the absence (A, B and C) or the presence (D, E and F) of 50 μM arachidonic acid at various concentrations of Ca^{2+}. xPKC I (A and D), xPKC II (B and E) and αPKC (C and F) were assayed in the absence of PS, DO and PMA (X) or in the presence of 8 μg/ml of PS (▲), 0.8 μg/ml of DO (□), 10 ng/ml of PMA (■), PS plus DO (●) or PS plus PMA (○). Where indicated by arrows, 0.5 mM EGTA was added instead of $CaCl_2$. Results are normalized to the maximum activity obtained in the presence of PS and DO.

Enzymatic Properties of xPKCs: In Fig. 2, kinetic properties of xPKC I and xPKC II are shown in comparison with the rat brain α-subspecies of PKC (αPKC), which is the most common subspecies present in mammalian tissues and cell types. Responses of xPKC I and II to PS, DO, and PMA at various concentrations of Ca^{2+} were very similar to each other and to those of αPKC (Fig. 2A-C). Since unsaturated fatty acids such as arachidonic, oleic, and linoleic acids are found to enhance the reaction velocity of mammalian PKC in a synergistic fashion with DO (20), the effect of arachidonic acid on xPKC was examined. Activation of αPKC by PS and DO was significantly enhanced by the addition of 50 μM arachidonic acid, especially at lower concentrations of Ca^{2+} (Fig. 2F). This was also the case in the activation of xPKC I and II (Fig. 2D and E). However, subtle

Table 1. Effects of diacylglycerols and various tumor promoters

	Protein kinase activity (%)		
	xPKC I	xPKC II	Rat αPKC
1,2-Dioleoylglycerol	100	100	100
1,2-Dioctanoylglycerol	95	89	102
1-Oleoyl-2-acetylglycerol	92	91	96
1,2-Dimyristoylglycerol	84	88	79
PMA	67	79	81
Teleocidin	56	52	51
Mezerein	38	31	30
Resiniferatoxin	0	5	2
4α-PDD	1	0	2
4β-PDD	78	57	70

Enzyme activity was assayed in the presence of 8 μg/ml PS, 0.1 mM $CaCl_2$ and 0.2 mg/ml of H1 histone. Diacylglycerols were used at 1.3 μM (0.8 μg/ml in 1, 2-dioleoylglycerol), and tumor promoters were used at 16.2 nM (10 ng/ml in PMA). Results are normalized to the activity obtained with 1, 2-dioleoylglycerol. 4α- and 4β-PDD; 4α- and 4β-phorbol 12,13-didecanoate, respectively.

differences were noted: Compared with xPKC I or αPKC, xPKC II was significantly activated by arachidonic acid alone. This activation was independent of Ca^{2+} concentrations. DO alone showed a stimulatory effect on αPKC when arachidonic acid was present (Fig. 2F), whereas the effect was only subtle for xPKC II (Fig. 2E) and not found for xPKC I (Fig. 2D). These results indicate that xPKC I and II have slightly distinct kinetic properties, although they are basically similar to the mammalian PKC.

Effects of diacylglycerols other than DO and various tumor promoters were also examined, and results are summarized in Table 1. Both xPKCs were activated significantly by several diacylglycerols and tumor promoters listed. Among the phospholipids tested, PS was the best activator for both xPKCs as well as for αPKC as shown in Table 2.

Selective Disappearance of xPKC II in PMA-treated Oocytes: Although xPKC I and II showed very similar properties as examined *in vitro*, the enzymes responded differently to PMA in oocytes. As shown in

Table 2. Effects of various phospholipids

	Protein kinase activity (%)		
	xPKC I	xPKC II	Rat αPKC
Phosphatidylserine	100	100	100
Phosphatidic acid	53	59	66
Phosphatidylinositol	16	17	27
Phosphatidyl-ethanolamine	6	0	4
Phosphatidylcholine	4	0	0

Enzyme activity was assayed in the presence of 0.8 μg/ml DO, 0.1 mM $CaCl_2$ and 8 μg/ml of the phospholipid under the standard conditions described in MATERIALS AND METHODS. H1 histone (0.2 mg/ml) was used as substrate. Results are normalized to the activity obtained with phosphatydylserine.

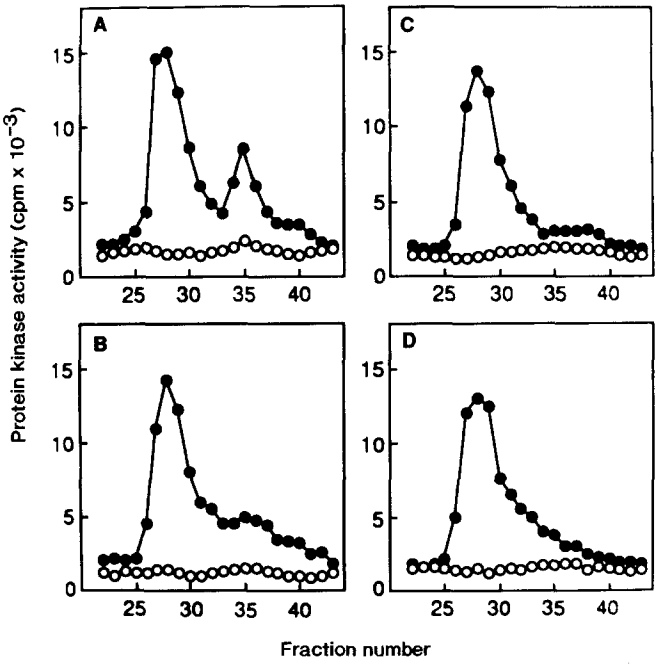

Fig. 3. Disappearance of xPKC II in PMA-treated oocytes. Full-grown (stage VI) oocytes were carefully selected by size from a pool of collagenase-treated oocytes and divided into four groups (4.8 ml each). Each group of oocytes was incubated with 300 nM PMA at 23°C for 0 min (A), 30 min (B), 1 hr (C) or 2 hr (D), then xPKC was purified. Elution profiles of the four hydroxyapatite column chromatography are shown. Assays were carried out in the presence of 8 μg/ml of PS, 0.8 μg/ml of DO and 0.1 mM $CaCl_2$ (●) or in the presence of 0.1 mM EGTA instead of PS, DO and $CaCl_2$ (○). $GVBD_{50}$ was about 3.5 hr under the same conditions.

Fig. 3, the activity of xPKC II decreased very rapidly when oocytes were treated with PMA. It disappeared almost within 1 hr (Fig. 3C), whereas the activity of xPKC I remained virtually unchanged up to 2 hr (Fig. 3D). Immunoblot analysis confirmed that the disappearance of the activity of xPKC II coincided with the disappearance of the 80 kDa polypeptide in the fraction.

There are several lines of evidence that PKC is involved in the insulin-induced and *ras*-mediated pathway for oocyte maturation, in which some protein kinases such as $p34^{cdc2}$ and S6 kinase are activated (22, 23). It is attractive to surmise that either the selective disappearance of xPKC II or the persistent existence of xPKC I may play some functions for the PMA-induced oocyte maturation. Isolation of xPKC described in this paper would facilitate to identify the responsible kinases and kinase cascade(s) for oocyte maturation.

ACKNOWLEDGMENTS

This work was supported in part by research grants (03151073, 02557015 and 03680221) from the Ministry of Education, Science and Culture, Japan.

REFERENCES

1. Nishizuka, Y. (1988) Nature **334**, 661-665.
2. Nishizuka, Y. (1986) Science **233**, 305-312.
3. Bement, W. M. & Capco, D. G. (1989) J. Cell Biol. **108**, 885-892.
4. Stith, B. J. & Maller, J. L. (1987) Expl. Cell Res. **169**, 514-523.
5. Smith, L. D. (1989) Development **107**, 685-699.
6. Varnold, R. L. & Smith, D. (1990) Development **109**, 597-604.
7. Kamata, T. & Kung, H.-F. (1990) Mol. Cell. Biol. **10**, 880-886.
8. Muramatsu, M.-A., Kaibuchi, K. & Arai, K.-I. (1989) Mol. Cell. Biol. **9**,831-836.
9. Chen, K.-h., Peng, Z.-g, Lavu, S. & Kung, H.-f. (1989) Second Messengers and Phosphoproteins **12**, 251-260.
10. Shambrook, J., Fritsch, E. F. & Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual (Cold Spring Harbor Lab., Cold Spring Harbor, NY)
11. Hashimoto, E., Takeda, M., Nishizuka, Y., Hamana, K. & Iwai, K. (1976) J. Biol. Chem. **251**, 6287-6293.
12. Ono, Y., Fujii, T., Igarashi, K., Kuno, T., Tanaka, C., Kikkawa, U. & Nishizuka, Y. (1989) Proc. Natl. Acad. Sci. USA **86**, 4868-4871.

13. Ono, Y., Fujii, T., Igarashi, K., Kikkawa, U., Ogita, K. & Nishizuka, Y. (1988) Nucleic Acids Res. **16**, 5199-5200.
14. Kishimoto, A., Saito, N., Ogita, K. & Nishizuka, Y. (1991) Methods Enzymol. **200**, 447-454 .
15. Sekiguchi, K., Tsukuda, M., Ase, K., Kikkawa, U. & Nishizuka, Y. (1988) J. Bichem. **103**, 759-765.
16. Ogita, K., Miyamoto, S.-I., Koide, H., Iwai, T., Oka, M., Ando, K., Kishimoto, A., Ikeda, K., Fukami, Y. & Nishizuka, Y. (1990) Proc. Natl. Acad. Sci. USA **87**, 5011-5015.
17. Wallace, R. A., Jered, D., Dumont, J. N. & Sega, M. W. (1973) J. Exp. Zool. **184**, 321-334.
18. Lohka, M. J. & Masui, Y. (1984) J. Cell Biol. **98**, 1222-1230.
19. Laemmli, U. K. (1970) Nature **227**, 680-685.
20. Shinomura, T., Asaoka, Y., Oka, M., Yoshida, K. & Nishizuka, Y. (1991) Proc. Natl. Acad. Sci. USA **88**, 5149-5153.
21. Barrett, C. B., Schroetke, R. M., Van der Hoorn, F. A., Nordeen, S. K. & Maller, J. L. (1990) Mol. Cell. Biol. **10**, 310-315.
22. Maller, J. L. (1990) Biochemistry **29**, 3157-3166.