Molecular and Cellular Biology, Nov. 1993, p. 6661–6666 Vol. 13, No. 11
0270-7306/93/116661-06$02.00/0
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Phosphatidylinositol 3-Kinase Activity Is Important for Progesterone-Induced *Xenopus* Oocyte Maturation

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Received 10 June 1993/Returned for modification 9 July 1993/Accepted 23 August 1993

In somatic cells, phosphatidylinositol 3-kinase (PI3 kinase) is a critical intermediary in growth factor-induced mitogenesis. We have examined the role of this enzyme in meiotic maturation of *Xenopus laevis* oocytes. PI3 kinase activity was present in immunoprecipitates of the p85 subunit of PI3 kinase from immature oocytes and markedly increased following progesterone stimulation. Injection of bacterially expressed protein corresponding to the C-terminal SH2 domain of p85 (SH2-C) inhibited progesterone-induced PI3 kinase activation and meiotic maturation. Injection of protein corresponding to the N-terminal SH2 domain or the SH3 domain of p85 did not inhibit PI3 kinase activation or maturation. SH2-C did not inhibit oocyte maturation induced by c-mos RNA injection. In addition, radiolabelled SH2-C was used to probe oocyte lysates, revealing that a novel 200-kDa protein bound to SH2-C. This protein may be an important mediator of progesterone-induced lipid metabolism in oocytes.

Phosphatidylinositol 3-kinase (PI3 kinase) is a cytoplasmic molecule that is important for signal transduction following growth factor stimulation of somatic cells (3, 4, 19). PI3 kinase is composed of two subunits: an 85-kDa subunit containing two Src homology 2 (SH2) domains and one Src homology 3 (SH3) domain (7, 29) and a 110-kDa subunit containing a kinase domain (15). The p85 subunit is thought to be important in linking the catalytic p110 subunit to activated growth factor receptors. The region of p85 that interacts with the activated platelet-derived growth factor β receptor (PDGF-βR) is the C-terminal SH2 domain (SH2-C) (21). The binding site of p85 on the PDGF-βR has been mapped to a small portion of the kinase insert region of the receptor (6, 20). Mutant PDGF-βRs lacking the p85 binding site do not mediate mitogenesis in response to PDGF stimulation (8). PI3 kinase has also been found to be an important signalling intermediary following stimulation of cells with nerve growth factor, epidermal growth factor, and insulin (1, 2, 3, 4, 31). Whether PI3 kinase enzymatic activity is increased following growth factor stimulation of cultured cells or whether binding of PI3 kinase to growth factor receptors simply redistributes PI3 kinase activity to the cell membrane in proximity to its substrates is a subject of controversy. Given the importance of PI3 kinase in somatic cell signal transduction and mitogenesis, we examined the role of this molecule in meiotic maturation, using *Xenopus laevis* oocytes.

Immature *Xenopus* oocytes are arrested at prophase of meiosis I. When they are stimulated in vivo with progesterone, they undergo a series of metabolic alterations, known as maturation, culminating in completion of meiosis I and progression to metaphase of meiosis II, when they are again arrested until fertilization (30). Several cytoplasmic kinases are activated following progesterone stimulation, including mos kinase (28), Raf-1 kinase (24), MAP kinase (9, 26), and $p34^{cdc2}$ (5, 9, 14, 16). The cell surface oocyte progesterone receptor has not been cloned; however, several investigators have demonstrated that progesterone stimulation of oocytes results in a rapid decrease in cyclic AMP (cAMP) levels and also reduces protein kinase A activity (10, 17, 23, 27). The precise role, if any, of decreased cAMP levels in the eventual activation of mos, Raf, MAP kinase, and $p34^{cdc2}$ is unclear. Recently, it was reported that receptors that utilize G_i-protein-mediated signal transduction pathways can stimulate PI3 kinase activity in human myeloid-derived cells (32). We speculated that PI3 kinase may be activated by progesterone stimulation of oocytes and that PI3 kinase may be an important early regulator of oocyte maturation. In this study, we determined that PI3 kinase is present in oocytes and that PI3 kinase activity is stimulated after progesterone stimulation. We also determined that blockade of PI3 kinase activation inhibits progesterone-induced meiotic maturation.

MATERIALS AND METHODS

Plasmid constructions and protein expression. DNA fragments corresponding to single murine p85 domains (SH2-C, SH2-N, and SH3) were subcloned into the T7 bacterial expression plasmid pHB40P as described elsewhere (21, 33).

In brief, the T7 expression vectors were propagated in bacterial strain BL21(DE3)plysS for production of the respective p85-derived proteins. For protein expression, bacterial cells were grown in dYT medium (21) containing ampicillin (100 μg/ml), induced with isopropyl-β-D-thiogalactopyranoside (IPTG), and then harvested and frozen as described elsewhere (21, 33). Lysis buffer {50 mM Tris-HCl [pH 7.5], 50% sucrose, 5 mM EDTA, 5 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid], 0.25% Nonidet P-40, 10 μg of aprotinin per ml, 10 μg of leupeptin per ml, 2 mM dithiothreitol, and 2 mM phenylmethylsulfonyl fluoride} was added to thawed cells, and lysates were sonicated. Extracts were cleared by centrifugation for 15 min at 16,000 × *g* at 4°C.

Protein concentration of extracts was determined by Coomassie blue staining and Western blotting (immunoblotting). Extracts were diluted to a final protein concentration of approximately 10 ng/μl in 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N′*-2-ethanesulfonic acid), pH 7.4.

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The *Xenopus* c-mos cDNA was a generous gift of D. J. Donoghue (University of California, San Diego).

Antibodies. The rabbit anti-p85 polyclonal antibody 1176 was raised against a p85 fragment spanning amino acids 1 to 282 of the murine sequence. The murine anti-p85 monoclonal antibody was raised against a peptide containing amino acids 608 to 724. The murine anti-MAP kinase monoclonal antibody was obtained from Zymed, Inc. The rabbit anti-mos polyclonal antibody was obtained from Santa Cruz Biotechnology, Inc. The murine monoclonal antiphosphotyrosine antibody was produced in our laboratory.

Oocyte injections. Fully grown oocytes were obtained from mature *X. laevis*. Oocytes were defolliculated by collagenase treatment (Sigma type II; 1 mg/ml) and maintained at 18°C in modified Barth's saline solution with HEPES, pH 7.6, with bovine serum albumin, Ficoll 400 (Sigma), penicillin G, and streptomycin added as described elsewhere (34). Oocytes were injected with 10 to 15 nl of protein solution and incubated at 18°C for various periods of time. Some oocytes were stimulated with 2 μg of progesterone per ml (Sigma).

Oocyte lysates. Oocytes were lysed by vigorous aspiration with a pipette tip in ice-cold NP40 lysis buffer (50 mM NaCl, 50 mM NaF, 30 mM sodium pyrophosphate, 5 mM EDTA, 10 mM Tris [pH 7.4], 0.25% Nonidet P-40, 2 mM phenylmethylsulfonyl fluoride, 25 μM leupeptin, and 0.2 U of aprotinin per ml). Ten microliters of lysis buffer was added per oocyte. Lysates were spun for 10 min at 16,000 × *g*, and clarified extracts were removed.

Western blotting. Oocyte lysates were boiled in Laemmli sample buffer (22), separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to nitrocellulose filters (Schleicher & Schuell). Filters were blocked in TBST (Tris-buffered saline with Tween 20) buffer containing 2% dried milk, as described elsewhere (21). Antibodies were added in TBST at appropriate dilutions. Bound primary antibody was detected with alkaline phosphatase-conjugated secondary antibody (Promega) and the appropriate color-developing reagents (Promega).

In vitro PI3 kinase assay. PI3 kinase immunoprecipitates were obtained by incubating oocyte lysate with polyclonal anti-p85 antibody 1176 at a dilution of 1:2,000 at 4°C for 12 h. Lysates were then incubated with protein A-Sepharose 4B beads for 30 min. Immunoprecipitates were washed twice in 0.5 M LiCl (with 10 mM Tris-HCl, pH 7.5) and once in 30 mM HEPES, pH 7.4. To assay for PI3 kinase activity, anti-SH3 immunoprecipitates were incubated in PI3 kinase buffer (30 mM HEPES, pH 7.4, 30 mM $MgCl_2$, 50 μM ATP, 200 μM adenosine, 0.2 mg of sonicated PI, 10 μCi of [γ-^{32}P]ATP [5,000 Ci/mmol]) for 20 min at room temperature. Reactions were stopped by the addition of 100 μl of 1 M HCl, and the phospholipids were extracted with 200 μl of a 1:1 mixture of chloroform-methanol. The reaction products were separated by thin-layer chromatography as described elsewhere (19). The conversion of PI to PI3 phosphate was determined by autoradiography or quantitated by scintigraphy of excised spots.

Histone H1 kinase assay. Oocyte lysates were used to assess maturation-promoting factor activity by an in vitro histone H1 kinase assay (30). Ten microliters of lysate was added to histone kinase buffer, and the mixture was incubated for 10 min at room temperature (25). Reaction mixtures were boiled in Laemmli sample buffer and separated by SDS–PAGE. Gels were stained with Coomassie blue, destained in acetic acid, dried, and evaluated by autoradiography. Bands corresponding to histone H1 were excised and quantitated by scintigraphy.

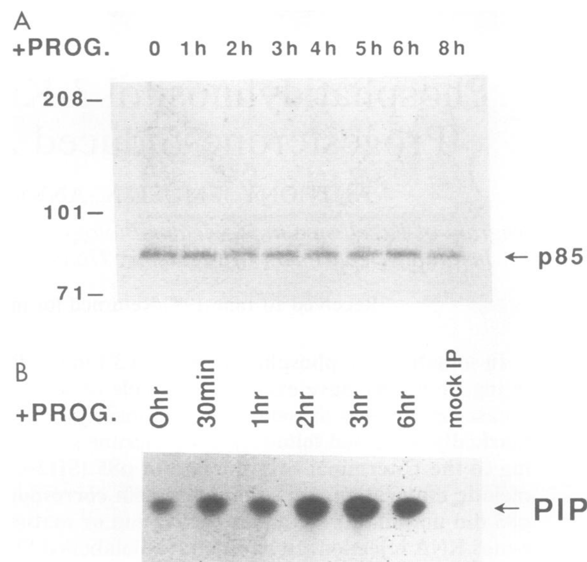

FIG. 1. (A) Detection of p85 in oocyte lysates. Oocytes were stimulated with 2 μg of progesterone per ml, and lysates were made at various time points. Proteins were separated by SDS–8% PAGE and transferred to nitrocellulose. Western blotting analysis was performed with a monoclonal anti-p85 antibody (F1A). (B) PI3 kinase specific activity increases following progesterone stimulation. Oocyte lysates were immunoprecipitated with a polyclonal anti-p85 antibody (1176) and were used in an in vitro PI3 kinase activity assay. Lysates were obtained at various time points following progesterone stimulation. For both panels A and B, 50% of oocytes exhibited GVBD after 5 h of progesterone stimulation. PROG, progesterone; IP, immunoprecipitation; PIP, phosphatidylinositol 3-phosphate.

Far-Western blotting. Oocyte lysates were separated by SDS-PAGE and transferred to nitrocellulose as described above. Proteins were denatured in 6 M guanidine–HCl (Sigma) and then renatured, and filters were blocked with 5% nonfat dry milk. Probes were generated with bacterially produced glutathione *S*-transferase (GST) fusion proteins containing the heart muscle kinase phosphorylation site (RRASV) (18). Recombinant proteins were purified with glutathione-agarose beads (Sigma) and labelled with [γ-^{32}P] ATP (5,000 Ci/mmol) (Amersham) and the catalytic subunit of heart muscle kinase (Sigma). Probes were eluted from the beads with 50 mM glutathione in 100 mM Tris-HCl, pH 8.0. Filters were hybridized overnight at 4°C with probe (250 kcpm/ml) in Hyb 75 buffer (20 mM HEPES [pH 7.7], 75 mM KCl, 2.5 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM EDTA, 0.05% Nonidet P-40, 1% nonfat dry milk), washed three times in Hyb 75 buffer, and autoradiographed.

RESULTS

The p85 subunit of PI3 kinase is present at constant levels during oocyte maturation. Monoclonal antibody F1A was raised against a portion of p85 corresponding to the SH2-C domain. Immature oocytes were obtained from adult frogs and were stimulated with progesterone (2 μg/ml) for several hours. Lysates were made from the oocytes at several time points and used in Western blotting analysis. A prominent 85-kDa band was recognized by the monoclonal anti-SH2-C antibody F1A (Fig. 1A). A markedly less intense band was noted at approximately 60 kDa with antibody F1A. The

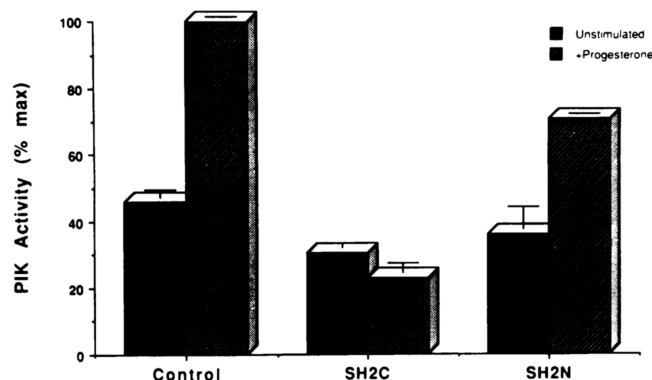

FIG. 2. SH2-C protein, but not SH2-N protein, blocks progesterone-induced PI3 kinase (PIK) activation. Oocytes were injected with approximately 100 pg of SH2-C or SH2-N protein fragments, and some were stimulated with progesterone. Lysates were obtained after 3 h, and p85 immunoprecipitates were used in an in vitro PI3 kinase activity assay. Spots corresponding to PI3 kinase products were cut out and analyzed by scintigraphy. Each column represents the average ± the standard error from duplicate assays.

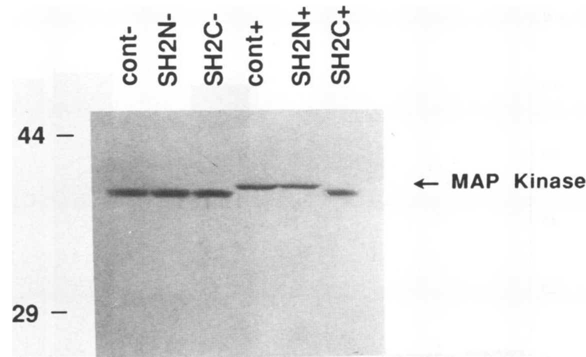

FIG. 3. SH2-C protein blocks progesterone-induced MAP kinase mobility shift. Oocytes were injected as described in the legend to Fig. 2, and some were stimulated with progesterone for 8 h. Lysates were fractionated by SDS-PAGE and analyzed by Western blotting with an antibody to MAP kinase (Zymed). Fifty percent of control oocytes exhibited GVBD after 4.5 h of progesterone stimulation. +, progesterone stimulation; −, no progesterone stimulation; cont, control.

85-kDa band did not vary in intensity or mobility with progesterone stimulation. This result suggests that the p85 subunit of PI3 kinase is present in *Xenopus* oocytes.

PI3 kinase activity increases following progesterone stimulation of oocytes. Given that p85 was found to be present in oocytes, we next investigated whether PI3 kinase is activated following progesterone stimulation. PI kinase assays (see Materials and Methods) were performed with immobilized PI3 kinase on protein A-Sepharose 4B beads and phosphatidylinositol as the substrate. Polyclonal antibody 1176 was raised against amino acids 1 to 282 of murine p85, overlapping the SH3 domain. This antibody was found to be efficient at immunoprecipitating murine PI3 kinase in vitro. When the PI kinase assay was performed with progesterone-stimulated oocytes, an early (after 30 min) modest increase in activity was noted (Fig. 1B), followed by a more marked (2- to 10-fold) increase in activity after 2 to 3 h of stimulation (Fig. 1B). Progesterone-stimulated oocyte lysates were also immunoprecipitated with antibody 1176 and blotted with monoclonal antibody F1A (anti-p85), revealing that equal amounts of p85 were immunoprecipitated at each time point (data not shown).

Injection of a fragment of p85 containing one SH2 domain, SH2-C, blocks progesterone-induced PI3 kinase activation in oocytes. Previously, it was shown that a protein fragment containing one SH2 domain of murine p85 (SH2-C) can block association of PI3 kinase with the activated PDGF-βR, whereas protein fragments containing only the SH3 domain or the SH2-N domain do not block this association (21). We therefore tested the ability of this fragment to block PI3 kinase activation in oocytes following progesterone stimulation. Immature oocytes were injected with SH2-C or SH2-N protein fragments and were immediately stimulated with progesterone. When oocytes were injected with 1 to 1.5 ng of SH2-C or SH2-N protein, autolysis was often observed; however, when 100 to 150 pg of protein was injected, oocytes appeared healthy for several days. After 100 pg of SH2-C or SH2-N was injected, oocytes were stimulated immediately with progesterone and lysates were made 3 h later. Lysates were immunoprecipitated with antibody 1176 and were used to perform a PI kinase assay as described above. When injected oocytes were compared with uninjected control oocytes, it was found that SH2-C blocked progesterone-induced PI3 kinase activation while SH2-N did not block activation (Fig. 2).

Injection of SH2-C blocks progesterone-induced MAP kinase phosphorylation. When immature oocytes are stimulated with progesterone, MAP kinase undergoes a mobility shift after several hours (9, 26). This shift correlates with an increase in MAP kinase activity (24, 26). We considered the possibility that PI3 kinase activation could be upstream of MAP kinase activation. Oocytes were injected with 100 pg of SH2-C or SH2-N and stimulated with progesterone. After 8 h, lysates were made and used in Western blotting experiments with a monoclonal anti-MAP kinase antibody (Zymed). These experiments demonstrated that progesterone-induced MAP kinase mobility shift was blocked in oocytes injected with SH2-C but not in those injected with SH2-N (Fig. 3).

Injection of SH2-C inhibits progesterone-induced meiotic maturation. Following progesterone stimulation of immature oocytes, germinal vesicle breakdown (GVBD) (nuclear membrane dissolution) occurs as oocytes proceed through meiosis I. GVBD is often used as a marker of oocyte maturation (30). Immature oocytes were injected with 100 pg of SH2-C, SH2-N, or SH3; stimulated with progesterone; and observed for 8 h. GVBD was suggested by the appearance of a white spot on the animal pole of the oocyte. When oocytes were injected with SH2-C, progesterone-induced GVBD was markedly inhibited compared with that in uninjected controls or in oocytes injected with SH3 or SH2-N (Fig. 4A). This inhibition of GVBD in oocytes injected with SH2-C was significant by chi-square analysis with continuity correction (P = 0.0001) when compared with GVBD in control oocytes. After 11 h of progesterone stimulation, the difference in GVBD between oocytes injected with SH2-C and those injected with SH3 was less pronounced than it was at 8 h; however, it was still significant (52 versus 91% GVBD).

Another sign of oocyte maturation is the activation of maturation-promoting factor, which is composed of $p34^{cdc2}$ and cyclin (12, 13). One in vitro assay that correlates with maturation-promoting factor activity is the phosphorylation

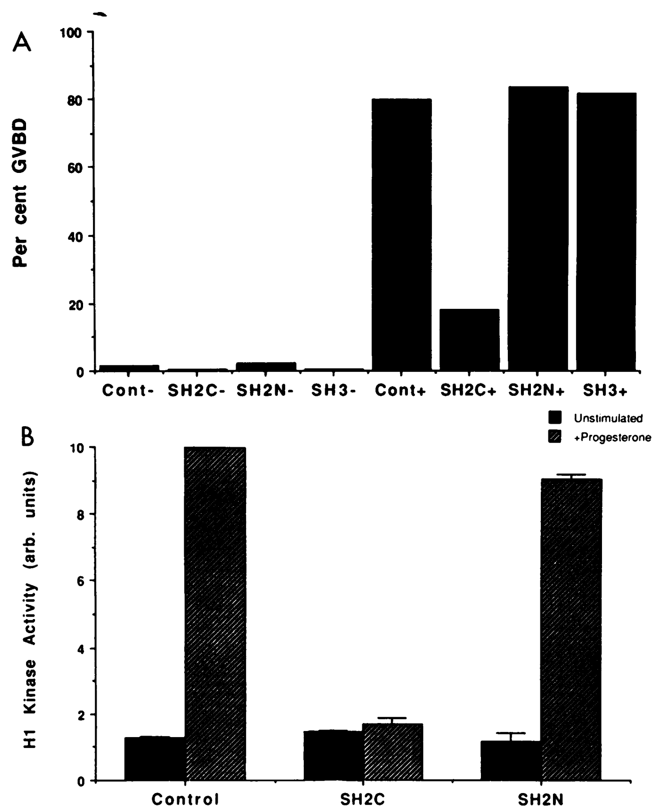

FIG. 4. (A) SH2-C protein blocks progesterone-induced GVBD. Oocytes were injected with SH2-C, SH2-N, or SH3 protein fragments, and some were stimulated with progesterone for 8 h. GVBD was assessed by the presence of a white spot in the animal pole. Each column represents at least 75 oocytes. – (leftmost four columns), oocytes not treated with progesterone; +, oocytes treated with progesterone; Cont, control. (B) SH2-C protein blocks progesterone-induced histone H1 kinase activation. Oocytes were injected with SH2-C or SH2-N protein fragments, and some were stimulated with progesterone for 8 h. Lysates were analyzed by an in vitro histone H1 kinase assay. Excised bands corresponding to histone H1 were analyzed by scintigraphy. Each column represents the average ± the standard error from duplicate assays. arb., arbitrary.

of histone H1 (30). Immature oocytes were injected with 100 pg of SH2-C or SH3 and stimulated with progesterone for 8 h, after which lysates were made. Lysates were utilized in an in vitro histone H1 kinase assay, which revealed that histone H1 kinase activity was blocked in SH2-C-injected but not SH3-injected oocytes (Fig. 4B).

Injection of SH2-C does not block maturation induced by injection of c-mos RNA. Previously, it has been demonstrated that injection of c-mos RNA or protein results in the maturation of oocytes (11, 35). mos expression occurs rapidly after progesterone stimulation. We hypothesized that PI3 kinase activation is upstream of mos activity following progesterone stimulation. When c-mos RNA and SH2-C were both injected, GVBD was observed in most oocytes after several hours (Fig. 5).

SH2-C binds to a 200-kDa protein from oocyte lysates in vitro. To determine the identity of potential PI kinase activators in oocytes, far-Western blotting was performed with a radiolabelled GST fusion protein containing the SH2-C protein fragment (GST–SH2-C) (18). A control GST fusion protein containing the breakpoint cluster region homology domain of p85 (GST-BCR) was also utilized. Unstimulated and progesterone-stimulated (4 h) oocyte protein lysates were separated by SDS-PAGE, transferred to nitrocellulose, and probed with a radiolabelled bacterially expressed protein fragment. Far-Western blotting revealed that GST–SH2-C bound most prominently to a 190- to 200-kDa protein from both unstimulated and stimulated lysates (Fig. 6). Previously, it has been shown that a tyrosine-phosphorylated peptide corresponding to residues 706 to 725 (19-mer) of the murine PDGF-βR (708/719 peptide) can block the association of p85 to the activated PDGF-βR in vitro (8). This peptide was used in a far-Western blot to determine the specificity of binding by GST–SH2-C. When far-Western blotting was performed with GST–SH2-C in the presence of 100 μM tyrosine-phosphorylated 708/719 peptide, the 200-kDa band was markedly reduced in intensity. On some blots, this band exhibited a mobility shift in progesterone-stimulated lysates. Reblotting of the nitrocellulose with antiphosphotyrosine antibodies demonstrated that the 200-kDa protein is tyrosine phosphorylated in both unstimulated and progesterone-stimulated lysates and that phosphotyrosine

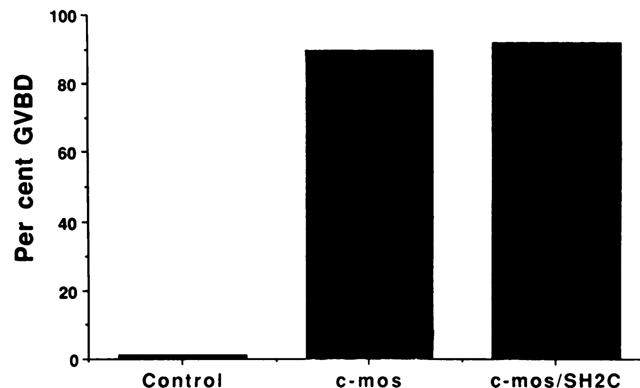

FIG. 5. GVBD in oocytes injected with c-mos RNA and SH2-C or with c-mos RNA alone. Each oocyte was injected with 10 ng of c-mos RNA and 100 pg of SH2-C protein or with 10 ng of c-mos RNA alone. Oocytes were observed for 8 h for the presence of a white spot on the animal pole.

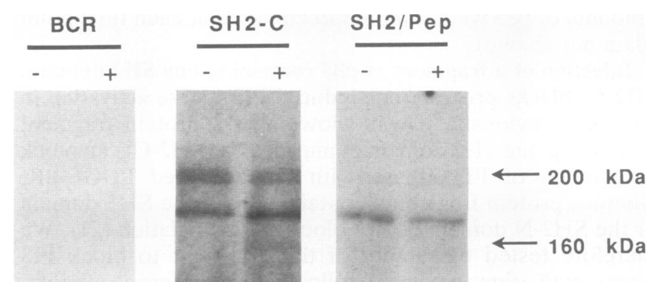

FIG. 6. Far-Western blot of unstimulated (–) and progesterone-stimulated (4 h) (+) oocyte lysates. Lysates were separated by SDS-PAGE, transferred to nitrocellulose, denatured, renatured, and probed with radiolabelled GST fusion proteins. Protein probes contained either the BCR or the SH2-C region of the p85 subunit of PI3 kinase. For the left panel, radiolabelled GST-BCR was used as a probe; for the middle panel, radiolabelled GST–SH2-C was used as a probe; and for the right panel, radiolabelled GST–SH2-C was used as a probe in the presence of 100 μM tyrosine-phosphorylated peptide 708/719 (corresponding to residues 706 to 725 of the murine PDGF-βR).

content does not increase with progesterone stimulation (data not shown).

DISCUSSION

Xenopus oocyte maturation is a complex biological process that involves the modification and activation of a variety of intracellular enzymes, resulting in the completion of meiosis I and the progression to metaphase of meiosis II. In this study, we have investigated the role of PI3 kinase in *Xenopus* oocyte maturation. In mammalian cultured cells stimulated with PDGF or other growth factors, PI3 kinase appears to be an early intermediary in the signal transduction pathway leading to mitogenesis. Although the proximal effects of PI3 kinase activity in mammalian cells remain uncertain, downstream effects of PI3 kinase activity may include the activation of Raf-1 kinase (8).

In this study, we determined that PI3 kinase was present in amphibian oocytes and that its activity increased following progesterone stimulation. Using p85 immunoprecipitates from oocyte lysates, we found that PI3 kinase activity increased 30 min after progesterone stimulation and peaked at 2 to 3 h. In growth factor-stimulated somatic cells, PI3 kinase rapidly associates with activated growth factor receptors; however, it is uncertain whether PI3 kinase is activated or merely relocated by activated receptors. Therefore, our results may provide the first clear demonstration of PI3 kinase activation in vivo.

We also demonstrated in this study that when injected into oocytes, a protein fragment containing one SH2 domain of the p85 subunit of PI3 kinase, SH2-C, blocked PI3 kinase activation by progesterone. We previously demonstrated that the SH2-C fragment is able to specifically block association of p85 with activated PDGF-βR in vitro (21). Injection of another protein containing a different SH2 domain of p85, SH2-N, did not block PI3 kinase activation by progesterone. This result is important because it implies that SH2-C specifically interacts with the upstream activator of PI3 kinase. Injection of oocytes with SH2-C also blocked progesterone-induced MAP kinase mobility shift, suggesting that PI3 kinase activity is upstream of MAP kinase activation in oocyte maturation.

Injection of immature oocytes with SH2-C inhibited progesterone-induced GVBD and histone H1 kinase activation, whereas injection with other protein fragments did not inhibit oocyte maturation. These results suggest that PI3 kinase activity is important for progesterone-induced oocyte meiotic maturation.

Injection of oocytes with both SH2-C and c-mos RNA resulted in GVBD in most oocytes after several hours. One interpretation of this result is that PI3 kinase is upstream of mos in the same signal transduction pathway leading to meiotic maturation. Further support for this interpretation comes from data presented here showing that SH2-C blocks progesterone-induced MAP kinase. Recently it has been demonstrated that mos activity is upstream of MAP kinase phosphorylation during oocyte maturation (24). It is possible, however, that mos is in a parallel pathway.

The mechanism by which PI3 kinase is activated in oocytes following progesterone stimulation remains unclear. In cultured mammalian fibroblasts, the SH2-C of p85 is thought to specifically interact with the kinase insert region of the phosphorylated PDGF-βR (6, 20, 21). One possibility is that in oocytes, the progesterone receptor activates an intracellular tyrosine kinase that, in turn, interacts with p85. To examine this possibility, we performed far-Western blotting with a radiolabelled GST fusion protein containing SH2-C as a probe. We demonstrated that SH2-C of p85 binds most prominently to a 200-kDa protein from unstimulated and progesterone-stimulated oocyte lysates. This 200-kDa protein is tyrosine phosphorylated in unstimulated and progesterone-stimulated oocytes. The identity and function of this protein are unknown at this time.

ACKNOWLEDGMENTS

We thank Daniel J. Donoghue (UCSD) for the *Xenopus* c-mos cDNA. We thank W. Michael Kavanaugh and Angus M. MacNicol for helpful discussions and critical review of the manuscript.

This work was supported by NHLBI Program of Excellence in Molecular Biology grant HL43821 and by NHLBI grant K11HL02571-01A1 (A.J.M.).

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