

## Regulation of Src kinase activity during *Xenopus* oocyte maturation

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### Abstract

Expression of constitutively active Src protein tyrosine kinase in *Xenopus* oocytes has been shown to accelerate oocyte maturation suggesting that Src may be involved in meiotic progression. However, meiotic regulation of endogenous Src kinase in oocytes has not been investigated in detail. To address this problem, we measured the activity, expression level, and phosphorylation state of the endogenous *Xenopus* Src (xSrc) and overexpressed xSrc mutants in the process of progesterone-induced oocyte maturation. We found that the enzyme is first transiently activated in the plasma membrane-containing fraction of oocytes within 3 min of progesterone administration. This event represents one of the earliest responses of oocytes to the hormone and should be related to triggering some early signaling pathways of maturation. Thereafter, xSrc activity increases again at the time of germinal vesicle breakdown (GVBD) and remains elevated till the completion of maturation. This elevation of xSrc activity is associated with a 2-fold increase of xSrc protein content in the absence of change in its specific activity and xSrc mRNA content. No significant changes in the phosphorylation state of C-terminal regulatory phosphotyrosine can be registered either in endogenous xSrc or in overexpressed kinase-negative and wild-type xSrc proteins during maturation. Altogether, these results indicate that upregulation of xSrc in the meiotic metaphase occurs at the translation level. We also demonstrate here that the expression of constitutively active xSrc in *Xenopus* oocytes is accompanied by the activation of mitogen-activated protein kinase (MAPK). Our data suggest that the Src kinase acts through the MAPK pathway to accelerate oocyte maturation.

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**Keywords:** Src kinase; *Xenopus*; Oocyte; Maturation; Meiosis

### Introduction

Fully grown oocytes of the African clawed frog *Xenopus laevis* are naturally arrested at the G2/prophase boundary of the first meiotic division. They have intact nuclear envelope, partially decondensed chromatin, abundant stock of mater-

nal mRNAs, and low activity of the key mitotic regulators, maturation-promoting factor (MPF, a complex of cyclin B and Cdc2 kinase) and cytosstatic factor (CSF), defined originally as a cytoplasmic activity from unfertilized eggs that causes metaphase arrest upon transfer into dividing embryos (Masui and Markert, 1971). Although a number of proteins have been implicated as CSF components, its exact molecular composition remains unknown. The *Xenopus* mitogen-activated protein kinase (MAPK) cascade, including Mos, MAPKK, MAPK, as well as MAPK downstream kinase p90<sup>Rsk</sup>, has been shown to play an important role in establishing CSF arrest in oocytes. Recently, the APC<sup>cde20</sup> inhibitor Emil has been identified as an essential component required for maintaining the CSF-arrested state

**Abbreviations:** CSF, cytosstatic factor; GVBD, germinal vesicle breakdown; IP3, inositol 1,4,5-trisphosphate; MAPK, mitogen-activated protein kinase; MPF, maturation-promoting factor; PI3K, phosphatidylinositol 3-kinase; PKA, cAMP-dependent protein kinase; PLC, phospholipase C; PR, progesterone receptor; RT-PCR, real-time polymerase chain reaction; SFK, Src family kinase; xSrc, *Xenopus* Src kinase.

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(Reimann and Jackson, 2002). The steroid hormone progesterone induces meiotic progression in *Xenopus* oocytes converting them into fertilization-competent eggs arrested at the metaphase of meiosis II. The finding that progesterone can initiate oocyte maturation only when applied externally, but not when injected into the cytoplasm or the nucleus, together with the fact that MPF activation can be induced in enucleated oocytes (Masui and Markert, 1971; Smith and Ecker, 1971) demonstrates unequivocally that the steroid acts at the plasma membrane independently of transcriptional changes. This action is different from action of the classical nuclear steroid receptor and suggests the existence of a nontranscriptional membrane-associated signaling receptor. One of the earliest responses to progesterone, a decrease in cAMP level due to partial inhibition of plasma membrane adenylate cyclase (Finidori-Lepicard et al., 1981; Schorderet-Slatkine et al., 1978), implicates a G-protein-coupled transmembrane receptor. The drop in cAMP concentration and subsequent decrease in cAMP-dependent protein kinase (PKA) activity are crucial for initiating maturation. Microinjection of PKA inhibitors induces oocyte maturation in the absence of progesterone, whereas injecting the PKA catalytic subunit blocks progesterone-induced maturation (Huchon et al., 1981; Maller and Krebs, 1977). Catalytically inactive PKA was also found to block progesterone- or Mos-induced oocyte maturation, suggesting a novel mechanism that may be related to sequestration of proteins rather than phosphorylating them (Schmitt and Nebreda, 2002). Another possible candidate for nontranscriptional membrane progesterone receptor is the classical nuclear progesterone receptor. Recently, a *Xenopus* homolog of nuclear progesterone receptor (xPR) has been cloned, whose overexpression increased sensitivity to progesterone and accelerated germinal vesicle breakdown (GVBD), Mos accumulation, and MAPK activation (Bayaa et al., 2000; Tian et al., 2000). Although initially this receptor could not be detected in the oocyte membrane, where progesterone signaling is originated, more recent finding demonstrates that a minor fraction of one form of the receptor (p82 xPR) is associated with the plasma membrane-containing fraction (Bagowski et al., 2001). Importantly, accelerated MAPK activation was also registered upon receptor overexpression in enucleated oocytes, implying that xPR can act through a nongenomic mechanism. Still, a great difference in EC<sub>50</sub> for transcriptional activation by the classical progesterone receptor and EC<sub>50</sub> for GVBD suggests that a progesterone-binding protein other than the classical receptor may be involved in progesterone-induced oocyte maturation.

Both G-protein-coupled receptors and classical progesterone receptor were shown to activate the Src/Ras/MAPK pathway.  $\beta\gamma$  subunits of G proteins can recruit phosphatidylinositol 3-kinase (PI3K)  $\gamma$  isoform to the membrane (Stoyanov et al., 1995) where they create phosphatidylinositol triphosphate docking sites for nonreceptor tyrosine kinase Src (Dikic et al., 1996; Lopez-Illasaca et al., 1997).

Then, activated Src kinase promotes MAPK activation through the assembly of a Ras-activating signaling complex. PI3K activity is increased in progesterone-treated oocytes and overexpression of constitutively active PI3K $\gamma$  is able to induce GVBD and stimulate MAPK (Hehl et al., 2001; Muslin et al., 1993). Evidence is presented that Ras is an essential component of oocyte maturation downstream of PI3K (Hehl et al., 2001; Hu et al., 1995). On the other hand, the classical progesterone receptor was found to contain a proline-rich motif that interacts with and activates Src family kinases (SFKs) by a SH3 domain displacement mechanism (Boonyaratanakornkit et al., 2001). Thus, it was also suggested to employ the Src/Ras/MAPK pathway (Migliaccio et al., 1998). Although activation of a plasma membrane-associated tyrosine kinase and low-level activation of MAPK have been registered in *Xenopus* oocytes shortly after progesterone application (Fisher et al., 2000; Morrison et al., 2000), neither the identity of progesterone-activated tyrosine kinase is established nor a pathway leading to MAPK activation is delineated.

In the present study, we investigated the activity and regulation of *Xenopus* Src kinase (xSrc) during meiotic maturation of *Xenopus* oocytes. A 57-kDa nonreceptor protein tyrosine kinase xSrc is a major SFK expressed at the protein level in *Xenopus* eggs (Sato et al., 1996); however, some other SFKs, such as Fyn, Yes, Looptail, and so forth, have been identified at the mRNA level (Steele et al., 1989a, 1990; Weinstein et al., 1998). *Xenopus* eggs were shown to contain two highly homologous copies of the *src* gene, presumably, due to the fact that the somatic genome of this species is tetraploid (Steele, 1985). Thus, xSrc represents a mixture of protein products encoded by *src1* and *src2* genes (Steele et al., 1989b). Recently, it has been established to play an important role in the processes of egg fertilization and activation (Sato et al., 1999, 2000b). Although microinjection of activated Src kinase was shown to accelerate progesterone-induced maturation of *Xenopus* oocytes (Spivack et al., 1984), the regulation of endogenous xSrc during oocyte maturation has not been investigated in detail.

Here, we demonstrate that the xSrc activity is transiently elevated in the plasma membrane-containing fraction of *Xenopus* oocytes within 3 min of progesterone treatment. Specific activity of the enzyme is also increased at that time, implying that xSrc activation takes place. xSrc activity grows again later in meiosis at the time of MAPK activation and GVBD. It is accompanied by a 2-fold increase of Src protein content in the absence of change in xSrc-specific activity. Overexpressing kinase-negative and wild-type forms of xSrc in resting and maturing oocytes demonstrated that, in contrast to mitotic Src regulation, no significant changes in the phosphorylation state of C-terminal regulatory tyrosine occur in meiosis. Also, the content of xSrc mRNA remains constant during maturation. These results indicate that upregulation of xSrc in meiotic metaphase takes place at the level of protein expression. We also demonstrate here that the expression of constitu-

tively active xSrc in *Xenopus* oocytes promotes low-level activation of MAPK in the absence of meiotic progression. The timing of MAPK activation suggests that the Src kinase acts through the MAPK pathway to accelerate oocyte maturation.

## Experimental procedures

### Materials

Female frogs of *X. laevis* were purchased from Hamamatsu Seibutsu Kyozaï (Hamamatsu, Japan). A synthetic cdc2 peptide, corresponding to residues 7–26 of the fission yeast cdc2 gene product, was synthesized as described previously (Fukami et al., 1993). Anti-xSrc polyclonal antibody was raised against a synthetic peptide corresponding to residues 410–428 of chicken c-Src as reported earlier (Sato et al., 1996). Polyclonal anti-MAPK and anti-pMAPK antibodies were from Cell Signaling (Beverly, MA), and anti-Src antibody clone 327 was from Oncogene (Boston, MA). Phosphotyrosine-specific antibodies anti-Src pY418 and anti-Src pY529 (corresponding to pY415 and pY526 in xSrc) were obtained from BioSource (Camarillo, CA), and anti-pCdc2 antibody was from Calbiochem (San Diego, CA). All anti-Src antibodies could recognize both *Xenopus* Src1 and Src2 proteins that have identical amino acid sequences in the regions of corresponding epitopes. Anti-mouse IgG rabbit polyclonal antibody was from Cappel (Aurora, OH) and alkaline phosphatase-conjugated goat polyclonal antibody against rabbit IgG was from Santa Cruz (Santa Cruz, CA). [ $\gamma$ - $^{32}$ P]ATP (6000 Ci/mmol) was purchased from Perkin-Elmer (Boston, MA). mMESSAGEmMACHINE RNA transcription kit and the RNase inhibitor SUPERaseIn were obtained from Ambion (Austin, TX). RNeasy purification kit was from Qiagen (Valencia, CA). SuperScript Preamplification System for first strand DNA synthesis was from GibcoBRL (MD, USA). Prostar ULTRA HF system for real-time polymerase chain reaction (RT-PCR) and pfu DNA polymerase were from Stratagene (CA, USA). SYBR green and TaqMan master mix kits for real-time PCR detection were from Applied Biosystems. Progesterone and poly (Glu–Tyr) were obtained from Sigma (St. Louis, MO). PP1, PP2, and PP3 were from Calbiochem. DEAE-cellulose (DE52) was purchased from Whatman (Kent, UK) and protein A-Sepharose was from Amersham Pharmacia. Other chemicals were from Nacalai Tesque (Kyoto, Japan), Wako (Osaka, Japan), or Sigma.

### Preparation of *Xenopus* oocytes

To obtain oocytes, frogs were anesthetized on ice followed by rapid decapitation, then ovaries were surgically removed and placed into OR-2 solution containing 82.5 NaCl, 2.5 mM KCl, 1 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 1 mM

Na<sub>2</sub>HPO<sub>4</sub>, 5 mM HEPES, pH 7.8. Ovaries were manually dissected into clumps of 50–100 oocytes and extensively washed with OR-2 solution. Clumps of oocytes were treated with 0.5 mg/ml collagenase (280 U/mg) in OR-2 at 23°C for 3 h by shaking at 60 revs/min. Oocytes were extensively washed in OR-2 solution and left for stabilization over 4 h. Undamaged defolliculated oocytes of stage VI were manually selected. Maturation was induced by addition of 10  $\mu$ M progesterone and monitored by occurrence of GVBD judged by appearance of a white spot on the animal hemisphere of oocytes. Presence of germinal vesicle was also confirmed by its detection in dissected oocytes after their fixation in 10% TCA. Quantitative injection of Src mRNA into oocytes was made under microscopic observation with a pulse-directed injector system (Drummond, Nanoject). RNA solutions were loaded into oil-filled glass microcapillary needles with a tip diameter of 10–30  $\mu$ m. About 50 ng of RNA dissolved in RNase-free water was injected per oocyte in a total volume of 50 nl. Samples of oocytes were frozen in liquid nitrogen and stored at –80°C until use.

### Plasmids and mutagenesis

*Xenopus scr2* gene was amplified using mRNA isolated from *Xenopus* liver with RNeasy RNA purification kit. First-strand cDNA was synthesized with SuperScript Preamplification System and polymerase chain reaction (PCR) was performed with pfu DNA polymerase using the sense primer 5'-AAAAGATCTAGGGCCCATGGGTGCCACTAAAAGCAAGCC-3' and the anti-sense primer 5'-AAAGGTACCGGGCCCTAAAGGTTGTCCC-CAGGCTGGTA-3'. The amplified full-length xSrc DNA fragment was inserted into the pCR-TOPO vector (pCR/xSrc) that was used to generate xSrc mutants by PCR-based methods. Constitutively active xSrc (xSrcKA) had Y526F mutation at the C-terminal site of regulatory phosphorylation. It was generated using the sense primer 5'-TTT-GGAATTCTCCTGACCGAGCTCACCACC-3' and the anti-sense primer 5'-AAAGGTACCGGGCCCTAAAGGTTGTCCC-CAGGCTGGTA-3'. The kinase-negative form (xSrcKN) was mutated at ATP binding site (K294M). It was produced with the sense primer 5'-CCACTCGAGTGGC-CATCATGACTCTGAAGC-3' and the anti-sense primer 5'-AAAGGTACCGGGCCCTAAAGGTTGTCCC-CAGGCTGGTA-3'. All xSrc mutants contained R121H substitution at mAb327 antibody recognition site that was generated with the primers 5'-CTGGTGGTTGGCA-CATTCCTAAGCTCTG-3' and 5'-CAGAGCTTAGG-GAATGTGCCAACCACCAG-3'. This substitution rendered xSrc immunoreactive with the mAb327 antibody. In detail, identification of mAb327 antibody recognition determinant will be described elsewhere. All xSrc mutants were subcloned into *Apa*I site of pBluescript II (SK–) vector (Toyobo) with the upstream T7 promoter to allow in vitro RNA synthesis.

### *In vitro mRNA synthesis*

In vitro RNA transcription coupled with capping by 7-methyl-guanosine at the 5' end was performed from *SpeI*-linearized pBluescript plasmid using mMESSAGEMMA-SHINE T7 high-yield capped RNA transcription kit, according to the manufacturer's manual. Purification of the synthesized message was carried out with RNeasy purification kit. Posttranscriptional polyadenylation of synthetic mRNA at the 3' end with *Escherichia coli* poly(A) polymerase was done according to the described protocol (Worthington, 1991). After purification with RNeasy purification kit, RNA was dissolved in RNase-free water at 1 mg/ml, which solution was used for microinjections into oocytes. RNA quality was controlled by denaturing agarose/formaldehyde electrophoresis performed essentially as described earlier (Sambrook and Russell, 2001).

### *Real-time PCR*

Total mRNA was isolated from *Xenopus* oocytes using RNeasy RNA purification kit and first-strand cDNA was generated with Prostar ULTRA HF RT-PCR system using poly(dT) or random primers. Real-time PCR (RT-PCR) was carried out with the use of ABI PRISM 7000 thermocycler (Applied Biosystems). The sense primer 5'-CTGCTTG-TCTGTCTCTGAC-3' and the anti-sense primer 5'-GTT-CCAAGTGCCCATCCAA-3' were used to detect xSrc DNA by SYBR Green detection system. An independent set of primers, the sense primer 5'-GCAAACACGCA-GATGGCCTA-3' and the anti-sense primer 5'-CCAAAG-CAGCCCTGTCCAAG-3' with the TaqMan probe 5'-CACCGCCTGACCGCTGTTTGTCCC-3', was employed with TaqMan detection system. In both approaches, the GAPDH gene was used as a housekeeping gene.

### *Partial purification of xSrc from the particulate fraction of oocytes*

The procedure was carried out essentially as described earlier (Sato et al., 1996). Briefly, oocytes in a packed volume of 1 ml were mixed with 3 ml of homogenization buffer containing 20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 10 mM  $\beta$ -mercaptoethanol, 1 mM sodium vanadate, 10  $\mu$ g/ml leupeptin, 20  $\mu$ M APMSF, and disrupted in a Teflon glass homogenizer. The homogenate was centrifuged at  $15,000 \times g$  for 15 min and the supernatant was further clarified by centrifugation at  $100,000 \times g$  for 1 h. The pellet was washed once again with the homogenization buffer by centrifugation at  $100,000 \times g$  for 30 min. Resulting pellet fraction was solubilized in 1 ml of homogenization buffer containing 1% Triton X-100 (buffer HB+). After centrifugation at  $100,000 \times g$  for 30 min, the supernatant was applied on a DEAE-cellulose column (0.5  $\times$  1 cm) equilibrated with the same buffer. The column was washed with buffer HB+ and

xSrc-containing protein fraction was eluted with 0.5 ml of 100 mM NaCl in the same buffer. Samples were dialyzed against buffer HB+ and stored at  $-20^\circ\text{C}$  until use.

### *Protein kinase assay*

Tyrosine kinase activity of xSrc-containing detergent soluble particulate fraction of oocytes was measured with poly (Glu-Tyr), as a protein substrate, and the activity of immunoprecipitated xSrc was estimated with a synthetic cdc2 peptide corresponding to amino acids 7–26 of the fission yeast cdc2 gene product. The reaction mixture of protein tyrosine kinase assay (20  $\mu$ l) contained 50 mM Tris-HCl, pH 7.5, 5 mM  $\text{MgCl}_2$ , 1 mM dithiothreitol, 1 mg/ml poly (Glu-Tyr), 2  $\mu$ M [ $\gamma$ - $^{32}\text{P}$ ]ATP (2  $\mu$ Ci), and 5–10  $\mu$ l of DEAE eluates containing normalized amount of protein (5  $\mu$ g of total protein corresponding to about 10 oocytes) or 10  $\mu$ l of immunoprecipitates. Samples were incubated for 10 min at  $30^\circ\text{C}$  then kinase reaction was terminated by the addition of concentrated SDS-PAGE sample buffer. Proteins were separated by SDS-PAGE (Laemmli, 1970) on 10% or 15% gels and radioactive bands of phosphorylated products were visualized and quantified by BAS2000 image analyzer (FUJI Film, Japan).

### *Immunoprecipitation*

Frozen oocytes were vortex mixed with 10 volumes of ice-cold homogenization buffer containing 1% Triton X-100 then sonicated for 2 min on ice with a TOMY UD-201 ultrasonic disrupter (Tomy Seiko, Tokyo). Samples were centrifuged for 10 min at 15,000 rpm then 200- $\mu$ l aliquots of supernatants were incubated with 10  $\mu$ g of anti-xSrc antibody (IgG fraction) raised against a synthetic peptide corresponding to the residues 410–428 of chicken c-Src for 2 h at  $4^\circ\text{C}$ . To collect immune complexes, protein A-Sepharose was added to the samples at a final concentration of 5% w/v for 1 h. Nonspecifically bound proteins were washed with the buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS. Finally, immune complexes were washed with tyrosine protein kinase reaction mixture lacking protein/peptide substrates and ATP.

### *Immunoblotting*

Twenty microliters of DEAE eluates was analyzed by immunoblotting with xSrc-specific antibodies in Figs. 3 and 4. In other experiments (Figs. 5, 6 and 7), 2–5  $\mu$ l of clarified oocyte homogenates (1:10 in buffer HB+) was taken for analysis. Protein samples were separated by SDS-PAGE on 10% polyacrylamide gels and transferred to PVDF membranes using a semidry blotting device (Bio-Rad). Membranes were blocked with T-TBS buffer (20 mM Tris-HCl, pH, 7.5, 150 mM NaCl, 0.05% Tween 20) containing 3 mg/ml bovine serum albumin and incubated at

room temperature for 2 h with 200-fold diluted polyclonal anti-xSrc, -pY418, -pY529, -pMAPK, -MAPK, and -pCdc2 antibodies or with 100-fold diluted monoclonal antibody mAb327. After treatment with the monoclonal antibody, the membranes were incubated with anti-mouse IgG rabbit polyclonal antibody at a 500-fold dilution for 1 h. All membranes were treated with a 1000-fold diluted alkaline phosphatase-conjugated goat polyclonal antibody against rabbit IgG. Membranes were thoroughly washed with T-TBS buffer and incubated in developing buffer (100 mM Tris-HCl, pH 9.5, 5 mM MgCl<sub>2</sub>, 100 mM NaCl, 50 µg/ml 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt, and 150 µg/ml nitro blue tetrazolium) to visualize the immune complexes.

#### Other methods

Protein content in the samples was determined spectrophotometrically using a protein assay kit (Bio-Rad). Mass spectrometric identification of xSrc as a mixture of Src1 and Src2 proteins will be described elsewhere (Iwasaki et al., unpublished data).

## Results

#### Overall profile of Src kinase activity during *Xenopus* oocyte maturation

xSrc activity is localized mainly in a particulate plasma membrane-containing fraction of *Xenopus* oocytes, as reported previously (Sato et al., 1996). Proteins of this fraction were solubilized in a detergent-containing buffer and subjected to DEAE-cellulose column chromatography, according to the Experimental procedures. After normalizing total protein content, protein kinase assay was performed with poly Glu-Tyr as a substrate in the presence of the Src-specific inhibitor PP2 or its inactive analog PP3 (Hanke et al., 1996). Both Src-specific (PP2-sensitive) and -nonspecific (PP2-insensitive) tyrosine kinase activity could be reliably detected by this method in the samples prepared from as few as 10 oocytes, with Src-specific activity normally exceeding the nonspecific one (Fig. 1A). Using this approach, we measured endogenous Src kinase activity in oocytes during progesterone-induced maturation and found that Src-specific tyrosine phosphorylation in the plasma membrane-containing fraction varied in the process of maturation, whereas Src-nonspecific tyrosine kinase activity remained virtually unchanged (Fig. 1B). Src-specific tyrosine kinase activity transiently increased about 2-fold within 3 min after progesterone administration, then fell down and remained at the level of untreated control until the time of MAPK activation and GVBD. At that time, Src-specific kinase activity increased again by about 2-fold and remained this elevated till the completion of maturation (Figs. 1B and C). A similar profile of xSrc kinase activity

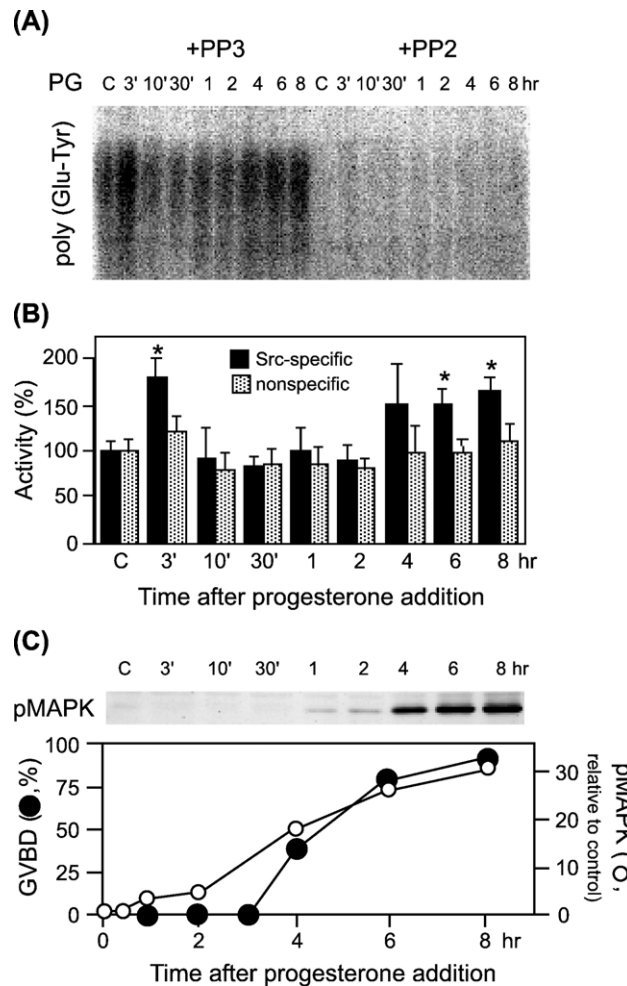


Fig. 1. xSrc activity in a plasma membrane-containing fraction of maturing *Xenopus* oocytes. A particulate fraction of maturing oocytes was obtained by ultracentrifugation of detergent-free homogenates. Proteins were solubilized in Triton X-100-containing buffer and subjected to DEAE-cellulose chromatography, as described in the Experimental procedures. Protein kinase activity eluted from the DEAE column at 100 mM NaCl was measured with the use of poly (Glu-Tyr), as a substrate of tyrosine kinases, in the presence of the Src family kinase specific inhibitor PP2 or its inactive analog PP3, both at 10 µM. Each sample of kinase assay contained 5 µg of solubilized particulate fraction protein corresponding to about 10 oocytes. After SDS-PAGE, radioactive bands of phosphorylated substrate were visualized and quantified by BAS2500 image analyzer (FUJI Film) (A and B). Panel C shows the time course of MAPK activation and GVBD after the addition of 10 µM progesterone to oocytes. Data of a representative experiment are shown in panels A and C. In panel B, data are means  $\pm$  SD of three independent experiments performed in duplicates with different batches of oocytes. Asterisk indicates a significant difference from the control ( $P < 0.05$ ).

was obtained in the kinase assay of the enzyme immunoprecipitated from detergent-soluble plasma membrane-containing fractions (see next section).

#### Immunoprecipitation kinase assay of xSrc

Evaluation of Src kinase activity by the above technique relied greatly on the specificity of the PP2 inhibitor.

Although PP2 was developed as a specific inhibitor of SFKs, it was also reported to inhibit the activity of some other tyrosine kinases (Bain et al., 2003). Moreover, PP2 does not discriminate between the different SFK members. Therefore, to confirm changes in Src activity during maturation, we performed protein kinase assay of xSrc kinase immunoprecipitated from detergent-soluble plasma membrane-containing fractions of *Xenopus* oocytes using the synthetic cdc2 peptide as an alternative substrate. High abundance of the enzyme in the reaction mixture of protein kinase reaction also allowed the detection of xSrc autophosphorylation in the immunoprecipitation assay. It should be noted, however, that the anti-peptide antibody against the activation segment of Src, which was used to immunoprecipitate xSrc kinase in these experiments, could also react with other SFK members, such as Yes, Fyn, and so forth. Nevertheless, mass spectrometry of the major immunoprecipitation product at 60 kDa could reveal only the presence of xSrc protein, but not other SFKs (Iwasaki et al., unpublished data), evidently due to the fact that xSrc represents the major SFK expressed at the protein level in *Xenopus* oocytes (Sato et al., 1996). Protein kinase activity of immunoprecipitates was greatly inhibited by the specific inhibitor of Src activity PP1 (Hanke et al., 1996), thus confirming that Src kinase constituted a major protein tyrosine kinase in the assay (Fig. 2A). Both autophosphorylation and phosphorylation of the Src-specific peptide

substrate in the immunoprecipitates increased about 2-fold within 3–5 min of progesterone administration and gradually declined afterwards (Fig. 2A). Later in maturation, Src kinase activity increased again by the time of GVBD and it remained elevated till the completion of maturation, as judged by both autophosphorylation and cdc2 peptide phosphorylation (Fig. 2B). These results are in good agreement with the data of Fig. 1 and confirm the two-phase character of xSrc activation during *Xenopus* oocyte maturation.

#### *Specific activity and tyrosine phosphorylation of xSrc during maturation*

Although the total protein content in the samples of kinase assay was normalized (5  $\mu$ g/assay), it was unclear whether the amount of Src protein in the assay was constant. Therefore, levels of Src protein in the samples of Fig. 1 were analyzed by Western blotting with an antibody raised against a synthetic peptide corresponding to the Src activation segment. Using this antibody, xSrc partially purified by DEAE-chromatography appeared on the blots as one major band usually accompanied by a minor doublet of a higher molecular weight. All these bands were considered as xSrc specific in the estimation of Src content in maturing oocytes. On the blots, total content of xSrc protein did not change significantly during the first 2 h of maturation, then increased about 2-fold after 4 h of progesterone administration at the time of GVBD and MAPK activation and remained this increased till the completion of maturation (Fig. 3A). On the other hand, xSrc-specific activity, calculated as a ratio of PP2-dependent tyrosine phosphorylation of poly (Glu–Tyr) in a radioactive kinase assay (data of Fig. 1) to xSrc protein content, was significantly increased at the time of early xSrc stimulation within 3 min after progesterone application but remained low at the time of GVBD (Fig. 3A). It was therefore concluded that activation of plasma membrane-associated xSrc occurs early upon progesterone treatment, whereas elevation of Src activity at GVBD is due to the increase of xSrc protein content.

To further investigate xSrc regulation during maturation, a phosphorylation site-specific antibody against C-terminal phosphoregulatory tyrosine has been employed. Immunoblotting with this antibody revealed that the increase in the total amount of xSrc at GVBD was accompanied by parallel changes in pY526 content. However, the extent of C-terminal tyrosine phosphorylation, as calculated by normalizing the content of phosphotyrosine to the total xSrc protein content, did not alter significantly during maturation (Fig. 3B).

#### *xSrc content in maturing oocytes*

Data presented above strongly suggested that a change in xSrc protein content, but not posttranslational modifi-

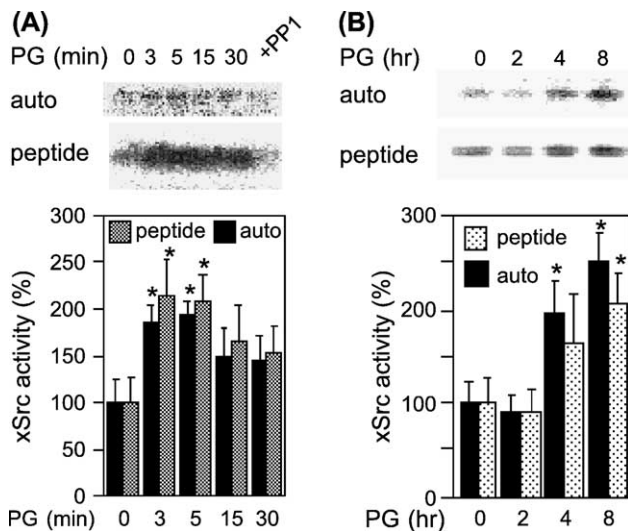


Fig. 2. Immunoprecipitation kinase assay of xSrc in maturing oocytes. Kinetics of early and late meiotic Src activation are shown in panels A and B, correspondingly. xSrc kinase was immunoprecipitated from a detergent-soluble particulate fraction of control and progesterone-treated oocytes with the antibody raised against Src activation segment. To determine Src-specific activity, Src autophosphorylation and phosphorylation of the Src-specific substrate cdc2 peptide were analyzed. Nonspecific protein kinase activity (panel A, right lane) was measured in the control immunoprecipitate from untreated oocytes in the presence of the Src family kinase-specific inhibitor PP1 (10  $\mu$ M). The results are representative of three experiments with different batches of oocytes. Bars in the lower panels are means  $\pm$  SD of three independent experiments performed in duplicates. Asterisk indicates a significant difference from the control ( $P < 0.05$ ).

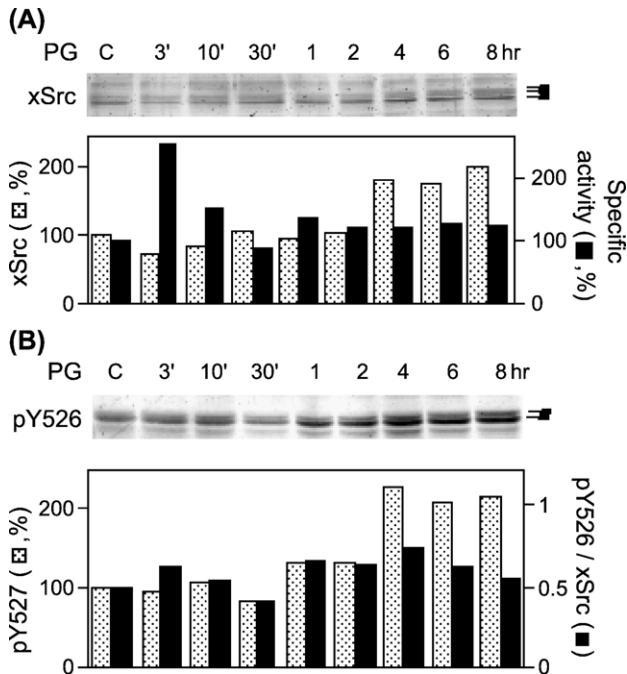


Fig. 3. Specific activity and site-specific tyrosine phosphorylation of xSrc during maturation. DEAE chromatography-purified particulate fraction of maturing oocytes was immunoblotted with anti-xSrc or anti-pY526 antibodies (panels A and B, correspondingly), as described in the Experimental procedures. Specific bands are denoted with arrows. Band intensity was estimated using Image Gauge V3.45 software (FUJI, Japan). Src-specific activity in panel A was calculated as a ratio of PP1-dependent tyrosine phosphorylation presented in Fig. 1 to xSrc protein content determined by immunoblotting. Relative band intensity in panel B represents a ratio of phosphotyrosine content in the C-terminal phosphorylation site to xSrc protein content. The results are representative of three experiments with different batches of oocytes.

cations, is responsible for the registered 2-fold increase of Src activity in meiotic metaphase. To address this point in more detail, the amount of xSrc in DEAE chromatography-purified samples of detergent-soluble particulate fraction obtained from several different batches of maturing oocytes was analyzed by immunoblotting with a Src-specific antibody. Although the initial content of xSrc in oocytes varied from batch to batch, the tendency to its increase at the time of GVBD persisted in all batches resulting in a clear statistical difference from the untreated control (Fig. 4A).

Next we estimated the content of xSrc mRNA in oocytes during maturation using the RT-PCR technique. Considering that a 2-fold difference in mRNA amount is sometimes difficult to register by this approach due to variation in parallel measurements, we synthesized two different sets of specific primers for two different parts of Src molecule (see Experimental procedures). In addition, two different detection techniques, such as TaqMan and SYBR Green, were used in RT-PCR experiments. These measurements could not reveal any statistically significant differences in xSrc mRNA content during maturation (Fig. 4B), implying that the increase in xSrc protein

content in meiotic M phase is regulated at the posttranscriptional level.

#### Expression of Src mutants in *Xenopus* oocytes

Src activation at mitosis was suggested to result from decreased phosphorylation on tyrosine 527 (Bagrodia et al., 1991). In contrast, our data presented in Fig. 3 showed that the increase in xSrc activity at meiotic M phase was not accompanied by changes in the extent of C-terminal tyrosine phosphorylation and Src-specific activity. To confirm this finding, the extent of C-terminal tyrosine phosphorylation was compared in resting and maturing oocytes overexpressing kinase-negative and wild-type xSrc proteins. Both proteins were expressed as single-band products that were heavily phosphorylated on C-terminal phosphotyrosine. Their expression levels exceeded those of the endogenous xSrc more than 10-fold, as judged by the relative band intensities of anti-pY526 immunoblot (Fig. 5A). Similar to endogenous xSrc, the content of pY526 in

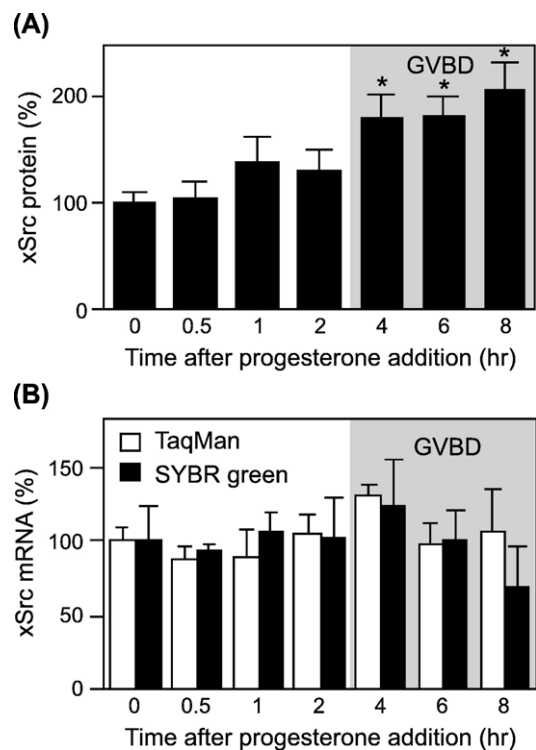


Fig. 4. Content of xSrc mRNA and protein in maturing oocytes. In panel A, relative content of xSrc in DEAE chromatography-purified particulate fraction obtained from several different batches of maturing oocytes was determined by immunoblotting with the anti-peptide antibody against the activation segment of Src. Data represent means  $\pm$  SD of five independent experiments. Asterisk indicates a significant difference from control ( $P < 0.05$ ). Panel B shows the relative content of xSrc mRNA in maturing oocytes measured by RT-PCR using TaqMan and SYBR green detection techniques with two different sets of primers. TaqMan data are means  $\pm$  SD of four measurements in two independent runs. Bars in SYBR green represent the range of duplicate samples in the same run. Data in the highlighted areas depict oocyte populations with more than 50% GVBD.

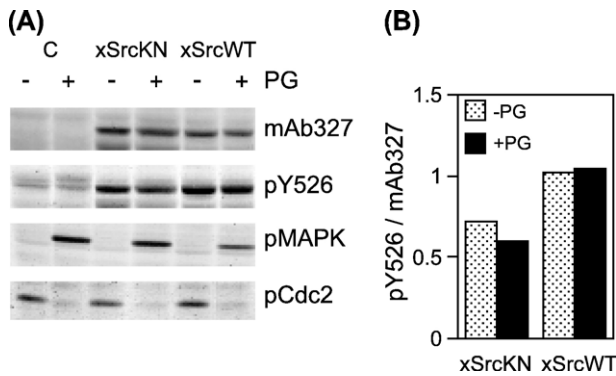


Fig. 5. Expression of kinase-negative and wild-type xSrc in resting and maturing oocytes. *Xenopus* oocytes were microinjected with kinase-negative (xSrcKN) or wild-type (xSrcWT) mRNA (40 ng/cell) and then incubated for 8 h at RT in the absence or presence of 10  $\mu$ M progesterone. In panel A, xSrc expression level and extent of C-terminal tyrosine phosphorylation were estimated by immunoblotting clarified oocyte extracts (0.5 oocyte/lane) with mAb327 and anti-pY526 antibodies, respectively. Completion of maturation was monitored by immunoblotting the extracts with anti-pMAPK and anti-pCdc2 antibodies. Panel B shows a ratio of C-terminal phosphotyrosine content to xSrc protein content.

overexpressed Src proteins was found to be the same in progesterone-matured and immature oocytes (Fig. 5B).

To test the ability of Src kinase to trigger meiotic progression, we also expressed a constitutively active form of xSrc protein with C-terminal tyrosine mutated to phenylalanine (xSrcKA) in oocytes. This enzyme displayed high autophosphorylation activity in the absence of C-terminal tyrosine phosphorylation, as judged by immunoblotting with the phosphorylation site-specific antibodies anti-pY415 and anti-pY526 (Fig. 6). Side-by-side expression of Src mutants revealed a slight mobility shift of xSrcKA protein band, evidently, due to the lack of C-terminal phosphorylation (data not shown). The single-band expression pattern of xSrc proteins alongside with the band mobility shift of constitutively active form implies that xSrc in oocytes is completely phosphorylated on C-terminal tyrosine. Analysis of xSrcKA expression in individual oocytes demonstrated that this mutant induced low-level MAPK activation in the absence of Cdc2 kinase dephosphorylation and cell cycle transition (Fig. 6). The magnitude of MAPK activation in xSrcKA-expressing oocytes was much lower than in progesterone-matured oocytes; MAPK band mobility shift could not be detected indicating that a major part of MAPK still remained unphosphorylated (i.e., inactive) in these cells. Although xSrcKA expression in oocytes failed to induce meiotic progression in the absence of progesterone treatment (Figs. 7C and D), it noticeably accelerated MAPK activation, Cdc2 kinase dephosphorylation, and GVBD in progesterone-treated oocytes (Figs. 7A, B, and D). Complete manifestation of these maturation markers in xSrcKA-expressing oocytes could be registered within 4 h of progesterone treatment. Importantly, not only the complete MAPK activation but also its onset could be detected much earlier in Src-expressing oocytes (both

progesterone treated and untreated) than in the control oocytes (see Discussion).

## Discussion

Although changes of Src activity in the mitotic cell cycle have been well documented and the regulatory mechanisms of Src activation in mitosis have been thoroughly investigated (Bagrodia et al., 1991; Chackalaparampil and Shalloway, 1988; Kaech et al., 1993), there were no detailed reports presented concerning Src regulation in the meiotic cell cycle. The fact that expression of constitutively active Src kinase in *Xenopus* oocytes can accelerate oocyte maturation (Spivack et al., 1984) suggested Src involvement in meiotic progression.

In the present study, we investigated Src regulation during progesterone-induced maturation of *Xenopus* oocytes. In oocytes, Src is maintained in a low-activity state (Sato et al., 1996) and it is first transiently activated in a detergent-soluble plasma membrane-containing fraction of oocytes within 3 min after induction of maturation by progesterone (Figs. 1 and 2). This increase in Src activity represents one of the earliest transcription-independent responses of oocytes to progesterone.

xSrc is suggested to play an important role in the transduction of fertilization signal that has been recently established to require sequential activation of Src family kinases, phospholipase C (PLC $\gamma$ ), and IP3 receptor of the

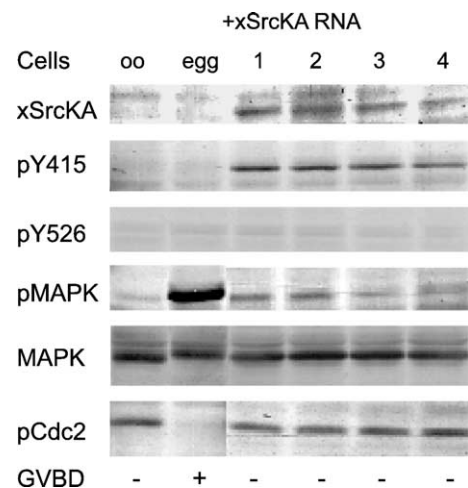


Fig. 6. Expression of constitutively active xSrc in individual oocytes. Clarified extracts (0.2 oocyte/lane) were obtained from control oocytes (oo), matured oocytes (egg), and four individual oocytes expressing constitutively active xSrc (numbers 1–4), then subjected to immunoblotting with indicated antibodies. Eggs were produced by in vitro maturation of control oocytes in the presence of 10  $\mu$ M progesterone for 8 h. Constitutively active xSrc was expressed in resting *Xenopus* oocytes as described in Fig. 5. GVBD was monitored by the appearance of a white spot on the animal hemisphere of oocytes and confirmed by microscopic detection of germinal vesicle in dissected oocytes after their fixation with TCA in a parallel experiment.



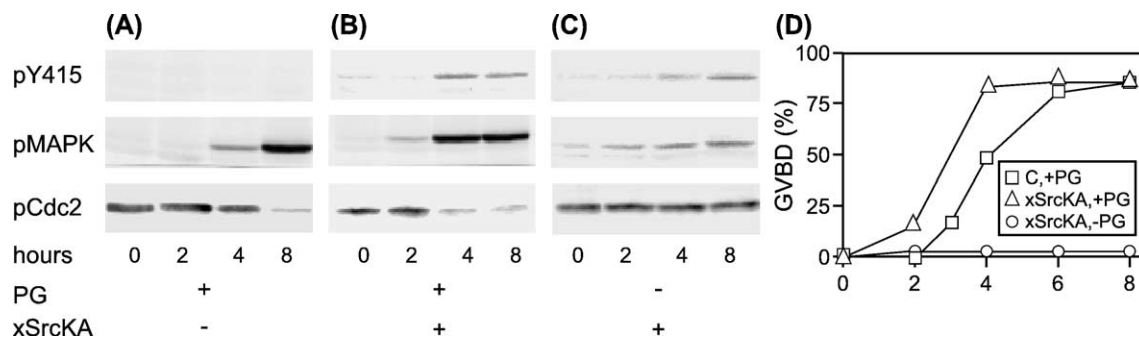


Fig. 7. Accelerated maturation of *Xenopus* oocytes expressing constitutively active xSrc. xSrcKA was expressed in *Xenopus* oocytes as described in Fig. 5. Clarified extracts were obtained from control maturing noninjected oocytes (A) and xSrcKA RNA-microinjected oocytes incubated in the presence (B) or absence (C) of 10  $\mu$ M progesterone for the indicated time. To monitor expression of xSrcKA and progression of maturation, the extracts (0.2 oocyte/lane) were immunoblotted with anti-pY415 xSrc, anti-pMAPK, and anti-pCdc2 antibodies. Panel D shows the time course of GVBD in progesterone-treated control oocytes ( $\square$ ) and xSrcKA RNA-microinjected oocytes in the presence ( $\triangle$ ) or absence ( $\circ$ ) of progesterone.

endoplasmic reticulum (Giusti et al., 2000; Sato et al., 2000a). Consistently, the Src kinase was shown to induce calcium release in *Xenopus* egg CSF-arrested extracts through PLC $\gamma$  and IP $_3$ -dependent mechanisms (Tokmakov et al., 2002b). Interestingly, progesterone too was reported to induce early stimulation of PLC and accumulation of IP $_3$  in *Xenopus* oocytes (Morrison et al., 2000; Stith et al., 1992). These events may be relevant to progesterone action, since reducing PIP $_2$  hydrolysis, IP $_3$ -receptor availability, or calcium gradients was shown to inhibit progesterone-induced maturation of *Xenopus* oocytes (Han and Lee, 1995). Furthermore, using a plasma membrane cortex preparation, progesterone has been demonstrated to act through a plasma membrane-associated tyrosine kinase to activate PLC in *Xenopus* oocytes (Morrison et al., 2000). Stimulation of tyrosine kinase activity could be detected within 10 min of progesterone administration. Although the identity of progesterone-stimulated tyrosine kinase is not established, tyrosine kinase activity seems to be important for the induction of maturation, microinjections of a tyrosine phosphatase or antibodies against phosphotyrosine inhibit this process (Hainaut et al., 1991; Tonks et al., 1990).

In the present paper, we identify membrane-associated Src kinase as a tyrosine kinase activated early upon progesterone treatment. Recently, progestins have been shown to promote rapid association of Src with the progesterone receptor resulting in Src activation in mammalian cells (Boonyaratanakornkit et al., 2001; Migliaccio et al., 1998). Also, Src downstream effector MAPK was reported to be activated in progesterone-treated mammalian cells (Migliaccio et al., 1998), suggesting that the classical progesterone receptor may transmit a hormone signal through the Src/Ras/MAPK pathway. In *Xenopus* oocytes, a transient low-level activation of MAPK has been registered shortly after progesterone application (Fisher et al., 2000), implying that the same signaling pathway also operates in oocytes. Early activation of MAPK in oocytes was suggested to occur through a Ras/Raf-dependent mechanism; however, at present, its role in the initiation of meiotic progression is not established.

In oocytes, MAPK is embedded in a loop of protein synthesis-dependent positive feedback that promotes MPF activation and meiotic progression (Ferrell, 1998; Tokmakov et al., 2002a; Tunquist and Maller, 2003). Microinjections of MAPK protein can induce complete MPF activation and meiotic progression in *Xenopus* oocytes in the absence of progesterone, whereas preventing MAPK activation inhibits or delays MPF activation and GVBD (Gotoh et al., 1995; Tokmakov et al., 1998). Although MAPK normally plays a major role in the progesterone-induced activation of MPF, recent results with the MEK inhibitor U0126 indicate that it is possible to induce MPF and GVBD in the absence of detectable MAPK activation (Gross et al., 2000). Also, MAPK inhibition by Pyst1 phosphatase delays but does not prevent initiation of maturation (Fisher et al., 1999). More recently, it has been found that blocking Mos synthesis by morpholino antisense oligonucleotides does not prevent progesterone-induced Cdc2 activation and GVBD; however, it substantially delays both events (Dupre et al., 2002). These findings demonstrate that although Mos synthesis and MAPK activation contribute considerably to maturation, they are dispensable for this process.

It has been reported earlier that the expression of deregulated highly active mutant of Src accelerated progesterone-induced maturation of *Xenopus* oocytes, increased content of tyrosine-phosphorylated proteins, and stimulated the activity of MAPK downstream S6 kinase RSK (Spivack et al., 1984; Unger and Steele, 1992). Consistently, we detected MAPK activation in *Xenopus* oocytes expressing constitutively active but not wild-type or kinase-negative xSrc (Figs. 6 and 7). However, the extent of MAPK activation was low, and GVBD, a morphological marker of meiosis I, did not occur in these oocytes, indicating that Src alone is not sufficient to promote complete maturation and some other pathways should cooperate in this process. Interestingly, on rare occasions, we detected GVBD in some batches of ultrasensitive oocytes expressing constitutively active xSrc in the absence of progesterone; however, these oocytes had a high rate of spontaneous maturation and

responded abnormally fast to progesterone, completing GVBD within 2 h (data not shown). Presumably, these batches of oocytes were obtained from hormonally compromised animals. Although MAPK activity in xSrcKA-expressing oocytes was much lower than that in matured eggs, it exceeded many fold the level of MAPK activity in resting oocytes (Figs. 6 and 7). Importantly, MAPK activation in oocytes expressing Src kinase in the absence of progesterone could be detected much earlier than MAPK activation in the course of normal maturation. Also, Src expression in progesterone-treated oocytes resulted in the accelerated onset of MAPK activation (Fig. 7). By promoting MAPK activation, Src kinase in maturing oocytes is embedded in the same loop of positive feedback that induces MPF activation and meiotic progression. Accordingly, accelerated dephosphorylation (activation) of Cdc2 kinase also takes place in maturing Src-expressing oocytes (Fig. 7). We suggest therefore that Src kinase acts through the MAPK pathway to accelerate oocyte maturation. More direct evidence for this comes from the fact that the MEK inhibitor U0126 abolishes the stimulatory effect of xSrcKA on maturation—it inhibits maturation of control and Src-expressing oocytes to the same extent (manuscript in preparation).

After the initial transient elevation at the beginning of progesterone treatment, Src activity returns to the control level and increases again at the time of GVBD in meiotic M phase (Figs. 1 and 2). Although this is reminiscent of mitotic Src activation, mechanisms underlying the increase of Src activity seem to be different in mitosis and meiosis. The fact that Src-specific activity does not change at GVBD indicates that no bona fide enzyme activation occurs in meiotic metaphase. Rather, the elevation of Src activity in meiosis is related to the increase of total Src protein content (Figs. 3 and 4). As a result, in matured oocytes arrested at the second meiotic metaphase (they can also be referred as unfertilized eggs), Src kinase is maintained in the same low-activity state as in immature G2 phase-arrested oocytes. This finding is in good agreement with previous observations indicating that Src kinase is inactive in *Xenopus* eggs and is transiently and robustly activated upon fertilization (Sato et al., 1996). On the other hand, mitotic activation of Src in mammalian cells was shown to result from decreased phosphorylation on C-terminal tyrosine (Bagrodia et al., 1991). However, in maturing *Xenopus* oocytes, we failed to detect any decrease in pY526 relative content in meiotic M phase (Fig. 3B). Also, the content of pY526 in overexpressed Src proteins was found to be the same in progesterone-matured and immature oocytes (Fig. 5B). Altogether, these results indicate that in contrast to mitosis, posttranslational phosphorylation on C-terminal tyrosine does not play an essential role in the meiotic regulation of Src activity.

At present, biological implications of Src activity changes in meiosis are not clear. Nongenomic Src activation immediately after induction of maturation may

be related to triggering some early signaling pathways of maturation, whereas stock-piling inactive Src protein later in meiotic metaphase may serve a purpose of increasing the robustness of fertilization signal mediated by Src activation. It may also be related to Src-mediated activation of some other signaling proteins that are involved in the regulation of meiotic progression. In this connection, Xp95 putative signal transduction protein recently cloned in *Xenopus* oocytes should be mentioned (Che et al., 1999). This protein was found to be phosphorylated from the first through the second meiotic divisions during progesterone-induced oocyte maturation. Remarkably, it can be phosphorylated by SFKs *in vitro* and expression of deregulated Src in *Xenopus* oocytes increases the abundance of tyrosine-phosphorylated Xp95, suggesting that Src can also regulate this protein during physiological oocyte maturation.

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