Identification and Cloning of Xp95, a Putative Signal Transduction Protein in Xenopus Oocytes

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A 95-kDa protein in Xenopus oocytes, Xp95, was shown to be phosphorylated from the first through the second meiotic divisions during progesterone-induced oocyte maturation. Xp95 was purified and cloned. The Xp95 protein sequence exhibited homology to mouse Rhophilin, budding yeast Bro1, and Aspergillus PalA, all of which are implicated in signal transduction. It also contained three conserved features including seven conserved tyrosines, a phosphorylation consensus sequence for the Src family of tyrosine kinases, and a proline-rich domain near the C terminus that contains multiple SH3 domain-binding motifs. We showed the following: 1) that both Xp95 isolated from Xenopus oocytes and a synthetic peptide containing the Src phosphorylation consensus sequence of Xp95 were phosphorylated in vitro by Src kinase and to a lesser extent by Fyn kinase; 2) Xp95 from Xenopus oocytes or eggs was recognized by an anti-phototyrosine antibody, and the relative abundance of tyrosine-phosphorylated Xp95 increased during oocyte maturation; and 3) microinjection of deregulated Src mRNA into Xenopus oocytes increased the abundance of tyrosine-phosphorylated Xp95. These results suggest that Xp95 is an element in a tyrosine kinase signaling pathway that may be involved in progesterone-induced Xenopus oocyte maturation.

Xenopus oocyte maturation is the process through which fully grown Xenopus oocytes (stage VI) mature into unfertilized eggs (1–3). Stage VI Xenopus oocytes are naturally arrested at the G2/M boundary of the first meiotic division. The G2/M block of stage VI oocytes is released by the steroid hormone progesterone, which initiates a series of events that lead to induction of the first and second meiotic divisions. The events that are instrumental for maturation induction include a decrease in cAMP-dependent protein kinase activity, synthesis of the c-Mos protein, activation of multiple signaling kinase proteins, including Mos protein kinase (4, 5), Raf protein kinase (6, 7), and MAP kinase (8–10), activation of Cdc25 phosphatase (11–13), and activation of multiple mitotic kinases. These mitotic kinases, including mitotic Cdc2 kinase (14, 15), MPM-2 epitope kinase (16), and polo-like kinase (17), induce meiotic divisions through the phosphorylation of a great number of proteins (18). The oocytes are then arrested at the second meiotic metaphase by cytoskeletal factor as mature oocytes or unfertilized eggs.

Fertilization triggers release of the metaphase arrest and induces onset of embryonic cell cycles (19).

Many of the components in the maturation induction pathway are still unknown. For example, it is unclear how Mos and Raf protein kinases are activated following progesterone stimulation; how activation of MAP kinase by Raf and Mos protein kinases leads to the activation of Cdc25 phosphatase; and how the phosphorylation orchestrated by multiple mitotic kinases induces meiotic divisions. Moreover, whereas tyrosine phosphorylation has been implicated in progesterone-induced Xenopus oocyte maturation (20, 21), neither the tyrosine kinases nor their substrates have been defined. Because most of these events involve protein phosphorylation, identification and characterization of novel proteins that become phosphorylated during progesterone-induced oocyte maturation may shed new light on this process.

We describe the identification, purification, and molecular cloning of Xp95, a novel 95-kDa protein in Xenopus oocytes which undergoes phosphorylation from the first through the second meiotic divisions. The protein sequence of Xp95 suggests that it belongs to an emerging family of signal transduction proteins, is a substrate of tyrosine kinases, and may interact with SH3 domain-containing proteins. In support of the possibility that Xp95 is a substrate of tyrosine kinases, we showed that Xp95 and an Xp95 peptide were phosphorylated in vitro by Src kinase and to a lesser extent by Fyn kinases. In addition, Xp95 from Xenopus oocytes and eggs was recognized by an anti-phosphotyrosine antibody, and the abundance of tyrosine-phosphorylated Xp95 increased during oocyte maturation. Furthermore, microinjection of deregulated Src message into Xenopus oocytes increased the abundance of tyrosine-phosphorylated Xp95. The features of Xp95 suggest that Xp95 functions in a signal transduction pathway involved in progesterone-induced oocyte maturation.

EXPERIMENTAL PROCEDURES

Preparation of Xenopus Oocyte, Egg, or Embryo Extracts—Interphase-arrested Xenopus oocyte extract and M phase-arrested Xenopus egg extract were prepared as described previously (16) with the follow-


dithiothreitol; EB, Xenopus egg extraction buffer; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis; TBST, Tris-based saline/Tween 20; PCR, polymerase chain reaction; ATPγS, adenosine 5'-O-(thiotriphosphate); d-Src, deregulated Src; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine

5522 This paper is available on line at http://www.jbc.org
ing modifications. *Xenopus* oocyte and egg extraction buffer (EB) was 80 mM β-glycerophosphate, 20 mM EGTA, and 15 mM MgCl₂, pH 7.3, supplemented with 20 mM NaF, 1 mM ATP, 1 mM DTT, 0.1 mM phenylmethylsulfonil fluoride, and 0.1 mg/ml each of leupeptin, pepstatin A, and chymostatin. Both extracts were immediately frozen at −70 °C. To thioisylate phosphorylated proteins in egg extract, the extract was precipitated with 40% ammonium sulfate, desalted by gel filtration through 10DG columns (Bio-Rad) into EB supplemented with 1 mM DTT and 50 μM ATP-S, and incubated at room temperature for 3–4 h.

To determine the timing of Xp95 gel mobility shift during progesterone-induced *Xenopus* oocyte maturation, surgically removed stage VI oocytes cultured in modified Barth’s solution (22) were treated with 1 μM progesterone. At 1-h intervals, six oocytes were collected and immediately homogenized by pipetting with 60 μl of EB supplemented 1 mM DTT and 0.1 mg/ml each of leupeptin, pepstatin A, and chymostatin.

The homogenate was then centrifuged at ~14,000 × g for 30 min, and the layer between the surface lipid layer and bottom yolk pellet was collected and immediately frozen at −70 °C.

To examine the Xp95 gel mobility shift after fertilization, egg laying was induced by injection of 800 units of human chorionic gonadotropin (Sigma) into the dorsal lymph sacs of adult female frogs. On the following day, sperm was prepared from testes surgically removed from adult male frogs and suspended in 0.1× modified Barth’s solution. Eggs were collected, fertilized with the sperm suspension, and then dejellied by staining with 0.1% Coomassie Blue R-250 in 1% acetic acid, 40% methanol followed by destaining with 7.5% acetic acid and 45% methanol followed by destaining with 25% acetic acid and 75% methanol. The Xp95 band was then excised and digested with CNBr in situ. The digested peptides were then separated by Tricine/SDS-PAGE (27), transblotted onto an Immobilon-P membrane, and visualized by Coomassie Blue staining. The most prominent band was excised from the membrane and sequenced in situ with an automated amino acid sequencer (model 473A, Applied Biosystems, Foster City, CA). In the alternative procedure, the gel-separated proteins were directly stained with 0.5% Coomassie Blue in 10% acetic acid and 45% methanol followed by destaining with 7.5% acetic acid and 45% methanol followed by destaining with 25% acetic acid and 75% methanol.

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Injection of RNA into Xenopus Oocytes and Examination of Xp95 Tyrosine Phosphorylation—Ovarian tissue was surgically removed from adult Xenopus laevis (Xenopus Express, Beverly Hills, FL) and anesthetized by 2.5 mg/ml 3-aminobenzoic acid ethyl ester (Sigma). Stage VI oocytes were manually defolliculated and maintained in modified Barth’s solution. Myc-Xp95 fusion cDNA was constructed by inserting Xp95 cDNA in frame between the EcoRI and StuI sites in the CS2+MT vector (28, 29). cDNA containing the coding region of deregulated version of Xenopus c-src gene has been previously described (20). RNAs for injection were produced by in vitro transcription of the linearized plasmids using Ambion mMESSAGE mMACHINE SP6 Large Scale In Vitro Transcription kit for Capped RNAs (Austin, TX). After the transcripts were purified by LiCl precipitation, oocytes were each microinjected with 2-3 ng of RNA in 10-20 nl of H2O or an equivalent amount of H2O and were maintained overnight (~12 h) in modified Barth’s solution. For the experiments that involved partial purification of Xp95 from injected oocytes by chromatography, 300 oocytes were injected with each sample, and the injected oocytes were extracted in an Eppendorf tube with 2 volumes of EB supplemented with 20 mM NaF, 1 mM sodium orthovanadate, 1 mM DTT, and 0.1 mg/ml protease inhibitors. The extracts were then precipitated with 40% ammonium sulfate and desalted into 0.25× EB by gel filtration through a 3-ml G-25 column. The desalted samples were absorbed with 200 μl of HiTrap Q-Sepharose resin in an Eppendorf tube at 4 °C for 10 min, and supernatant was collected after pelleting the resin by centrifugation. The supernatants were then absorbed with 300 μl of Affi-Gel blue resin at 4 °C for 20 min, and the resin was recovered. The Affi-Gel blue resins were then washed four times with 0.25× EB and eluted with 300 μl of SDS-PAGE sample buffer at 90 °C for 5 min. For the experiments that involved isolation of Myc epitope tagged Xp95 from injected oocytes, 60 oocytes were injected with each sample, and the injected oocytes were extracted in an Eppendorf tube with 4 volumes of EB supplemented with 20 mM NaF, 1 mM sodium orthovanadate, 1 mM DTT, and 0.1 mg/ml protease inhibitors. The extracts were then absorbed with 1 μg of anti-Myc polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) immobilized onto 10 μl of Affi-Prep protein A beads (Bio-Rad) at 4 °C for 3 h. After the protein A beads were pelleted down by centrifugation and washed five times with EB including 0.5 mM NaCl and 0.5% Nonidet P-40, the immobilized proteins were eluted by SDS-PAGE sample buffer.

RESULTS
Xp95 Undergoes a Phosphorylation during Progesterone-induced Oocyte Maturation—In the process of studying the mechanism that initiates Cdc25 phosphorylation during Xenopus oocyte maturation, we generated a polyclonal antibody against recombinant Xenopus Cdc25. The affinity purified anti-Cdc25 antibody recognized not only the 76-kDa Cdc25 but also a more prominent polypeptide of ~95 kDa designated Xp95 (Fig. 1A). Studies were initiated to investigate the molecular identity and possible functions of Xp95 due to the following observations.

Xp95 in interphase-arrested oocyte extract migrated as a single band in SDS-PAGE; however, in M phase-arrested egg extract it became a doublet, as shown in Fig. 1B. Since the appearance of a slower migrating species of Xp95 in egg extract is reminiscent of many proteins that become phosphorylated during progesterone-induced oocyte maturation, like c-Mos (5), c-Raf (7), p42MAPK (8, 30), Xnf7 (31), and Cdc25 phosphatase (11, 13, 32), the following experiments were carried out to determine whether the gel mobility shift of Xp95 was due to protein phosphorylation. First, we examined whether dephosphorylation could eliminate the slower migrating form of Xp95 in egg extract. Egg extract was immobilized onto nylon membrane discs and incubated either with control buffer or potato acid phosphatase, which accounts for 80% histone H1 kinase activity in Xenopus oocytes and eggs (35), is transiently activated at the first meiotic division, inactivated in the interphase between the first and second meiotic divisions, and activated again at the second meiotic division. As shown in Fig. 2A, the Xp95 doublet appeared at approximately the same time as MAP kinase activation and remained for the rest of the maturation process.
This was in contrast to Cdc2 kinase activity, which rose, fell, and rose again during this period. The gel mobility shift of Xp95 from the first to the second meiotic divisions suggests that the Xp95 phosphorylation is associated with progesterone-induced signal transduction but not with M phase induction.

The metaphase arrest of mature Xenopus oocytes or unfertilized eggs can be released by fertilization, which triggers dephosphorylation of many proteins phosphorylated during oocyte maturation. To determine the phosphorylation status of Xp95 after fertilization, Xenopus eggs were fertilized in vitro at time 0, and six embryos were extracted at 15-min intervals. Samples were processed as described for A.

Fig. 2. Timing of the Xp95 gel mobility shift. A, stage VI Xenopus oocytes were cultured in Barth’s solution containing 1 µM progesterone at 22 °C for up to 8 h, and six oocytes were extracted at 1-h intervals. Samples were immunoblotted with anti-Cdc25 antibody (top panel) and anti-Xenopus MAP kinase antibody (middle panel) and assayed for histone H1 kinase assay (bottom panel). B, Xenopus eggs were fertilized in vitro at time 0, and six embryos were extracted at 15-min intervals. Samples were processed as described for A.

Purification and Sequencing of Xp95—To investigate the molecular identity of Xp95, Xp95 was purified from oocyte extracts and its partial amino acid sequence determined. For this, we first fractionated oocyte extract consecutively by ammonium sulfate precipitation, Q-Sepharose chromatography, Affi-Gel blue chromatography, and sucrose gradient sedimentation as described under “Experimental Procedures.” As shown in Fig. 3A, when the partially purified Xp95 was separated by SDS-PAGE, the immunostained Xp95 coincided with a distinct silver-stained band indicating that Xp95 was now separated from the rest of the proteins by SDS-PAGE. Next, we isolated the Xp95 band from the gel and cleaved it by either proteolysis or cyanogen bromide treatment. Finally, three of the internal peptides were sequenced.

The three Xp95 peptide sequences consisted of 42, 17, and 11 amino acids. A search of protein data bases showed that none of them exhibited homology with Xenopus Cdc25 protein sequence; however, all exhibited a high degree of homology to Caenorhabditis elegans YNK1 (Fig. 3B). To explore further the homology between Xp95 and YNK1, we tested whether Xp95 was recognized by a polyclonal antibody raised against a YNK1 recombinant protein (24). As shown in Fig. 3C, the antibody recognized a single prominent band of 95 kDa in oocyte extract and a doublet in egg extract in the same region. The recognition was eliminated if the antibody was preincubated with the recombinant YNK1 protein (data not shown). The antibody also recognized partially purified Xp95 from oocyte extract (data not shown). The specific recognition of Xp95 by anti-YNK1 antibody, together with the sequence homology, indicates that Xp95 is closely related to YNK1.

Xp95 Belongs to a Family of Signal Transduction Proteins—To explore further the molecular identity of Xp95, a full-length cDNA encoding Xp95 was cloned by PCR-based method and Xp95 protein sequence deduced. As shown in Fig. 4, the cDNA contained 3506 base pairs consisting of both coding and noncoding regions. The coding region started with ATG in the context of a Kozak consensus sequence (37, 38) and ended with a termination codon followed by a poly(A) tail. The open reading frame encodes a protein of 867 amino acids with a calculated molecular mass of 96 kDa, which approximates the apparent molecular mass of Xp95 on SDS-PAGE. Northern blot analysis showed that this cDNA hybridized to a single transcript of ∼4 kilobase pairs (data not shown). These results indicate that this cDNA contains the entire coding sequence and most, if not all, of the noncoding sequences of the transcript.

A search of protein data bases with the Xp95 protein sequence for homologous proteins confirmed that Xp95 is homologous to YNK1. In addition, it revealed that Xp95 is also homologous to Aspergillus PalA (39), mouse Rhophilin (40), and budding yeast Bro1 (41). Whereas the function of YNK1 is presently unknown, the three other proteins have all been implicated in signal transduction (see “Discussion”), indicating that Xp95 belongs to a family of signal transduction proteins.

Pairwise sequence alignment by the BESTFIT program (Genetics Computer Group, University of Wisconsin, Madison, WI) showed that Xp95 is homologous to YNK1, Bro1, and PalA throughout the entire length and is homologous to Rhophilin through part of the length, i.e. amino acids 1–530 of Xp95 is homologous to amino acids 111–640 of Rhophilin (data not shown). This indicates that Xp95-related proteins fall into two subfamilies. Xp95 is 39% identical and 51% similar to YNK1, 27% identical and 39% similar to PalA, and 21% identical and 30% similar to Bro1. Thus, in the subfamily including Xp95, Xp95 is most closely related to YNK1.

Multiple sequence alignment of Xp95 with YNK1, Bro1, and PalA revealed three conserved features (Fig. 5). First, there were seven perfectly conserved tyrosines suggesting that Xp95 may be a substrate of tyrosine kinases. Second, one of the conserved tyrosines is in a highly conserved sequence context (KDNDFIYXXXV) which matches the phosphorylation consensus sequence (R/K)[EX2Y,3]/[D/E][EX2Y,3] for the Src family tyrosine kinases (42). This suggests that Src family tyrosine kinases may phosphorylate Xp95. Third, all four proteins contain a proline-rich region near their C terminus, which contains...
multiple potential SH3 domain-binding motifs PXXP (43). This raises the possibility that Xp95 may interact with SH3 domain-containing proteins.

Xp95 Is Phosphorylated by Src and Fyn Kinases—To investigate the possibility that Xp95 is a substrate of Src family kinases, we first examined whether Xp95 could be phosphorylated in vitro by Src or Fyn kinase. Xp95 was partially purified from Xenopus oocyte extract. Purified Src and Fyn kinases, free of contaminating kinase activities, were obtained from a commercial source and were shown to have comparable efficiencies when used to phosphorylate the standard substrate peptide KVEKIGEGTYGVYK (data not shown). Xp95 alone, each ki-
nase alone, or Xp95 plus each kinase was incubated at room temperature for 30 min in the presence of \[^{32}P\]ATP. All the samples were then immunoblotted with anti-YNK1 antibody and subjected to autoradiography. Results from immunoblotting with anti-YNK1 antibody showed that partially purified Xp95 contained both full-length Xp95 and a partially cleaved Xp95 at 70 kDa and that incubation with neither Src nor Fyn kinase changed the gel mobility of Xp95 (Fig. 6A). The autoradiography results showed that whereas incubation of Xp95 or each kinase alone generated no \[^{32}P\] labeling in the immunoreactive bands, incubation of Xp95 with either Src or Fyn kinases generated \[^{32}P\] labeling in both of the immunoreactive bands, although Src kinase generated stronger labeling than Fyn kinase (Fig. 6A). Quantitation of the radioactivity in the labeled bands by PhosphorImager analysis showed that the difference between Src and Fyn phosphorylation was 3.5-fold. These results indicate the following: 1) Xp95 is an in vitro substrate of both Src and Fyn kinases; 2) phosphorylation by Src kinase is more efficient; and 3) phosphorylation by Src or Fyn kinase is not sufficient to cause the gel mobility shift of Xp95.

Next, we examined whether Src and Fyn kinases could phosphorylate a synthetic peptide containing the conserved Src phosphorylation consensus sequence on Xp95. For this, two Xp95 peptides were synthesized by a peptide coupler as follows: one a 22-mer containing the Src phosphorylation consensus sequence (AAKKDNDFIYHDRVPDLKDLDP, amino acids 657-678) and the other a 19-mer (TPSPFPAISFLRPSQPST, amino acids 658-676). These peptides were incubated with Src and Fyn kinases, and the phosphorylation was analyzed by SDS-PAGE and autoradiography. The results showed that both Src and Fyn kinases phosphorylated the synthetic peptide, with Src kinase again being more efficient than Fyn kinase. This suggests that the phosphorylation sites on Xp95 are conserved and that Src kinase is a more effective phosphatase compared to Fyn kinase.

**Fig. 4. Sequence of Xp95 cDNA.** Nucleic acid sequence of a full-length Xp95 cDNA and the deduced amino acid sequence from the open reading frame are shown. The lower case and upper case type in the nucleic acid sequence indicate the noncoding and coding regions of the cDNA, respectively. The bold type sequence near the start codon ATG matches the Kozac consensus sequence, and the bold type in the 3’-noncoding region indicates the poly(A) tail.
310–321) and the other a 20-mer containing a different conserved tyrosine in a conserved context (ELVANLKEGTKFYNDLTDIL, amino acids 665–685). The two synthetic peptides were incubated with Src and Fyn kinases in the presence of \(^{32}\)P-ATP. Similar to the result obtained with Xp95 partially purified from oocyte extract, the 22-mer was \(^{32}\)P-labeled by both Src and Fyn kinases and the labeling was much stronger by Src kinase than by Fyn kinase (Fig. 6B). In contrast, no labeling was detected on the 20-mer by either Src or Fyn kinase. Quantitation of the radioactivity in the labeled bands by PhosphorImager analysis showed that the difference between Src and Fyn phosphorylation was 5-fold. These results suggest that Src and Fyn kinases phosphorylate Xp95 on the Src phosphorylation consensus sequence.

Both Src and Fyn kinase transcripts are detectable in *Xenopus* oocytes. To determine whether Xp95 was tyrosine-phosphorylated in vivo, we examined whether Xp95 in either oocyte or egg extract was recognized by PY99, a characterized monoclonal antibody that detects phosphotyrosine residues in proteins (44, 45). First, similar amounts of proteins from *Xenopus* oocyte and egg extracts were separated by SDS-PAGE and immunoblotted with PY99 to reveal all tyrosine-phosphorylated proteins. The same blot was also reprobed with anti-YNK1 antibody to reveal the position of Xp95. Both the single species of Xp95 in oocyte extract and the doublet form of Xp95 in egg extract coincided with staining by PY99. However, the signal was much stronger in the Xp95 doublet from egg extract than in the single species of Xp95 from oocyte extract (Fig. 7A).

Second, Xp95 was partially purified from *Xenopus* egg extract and immunoblotted in parallel with anti-YNK1 antibody and PY99. The doublet form of Xp95 in the partially purified sample again comigrated with a doublet recognized by PY99 (Fig. 7B). Third, Myc-epitope tagged Xp95 mRNA was microinjected into *Xenopus* oocytes that were subsequently incubated for 10 h in the presence or absence of progesterone. In the absence of progesterone, the injected oocytes remained immature, whereas in the presence of progesterone, \(95%\) of the injected oocytes matured after the 10-h incubation. The Myc-tagged Xp95 was then isolated from immature or mature oocytes by immunoprecipitation with anti-Myc antibody and the immunoprecipitated proteins were analyzed by SDS-PAGE and subjected to autoradiography.

**Fig. 5.** Conserved features of Xp95. The Xp95 protein sequence was aligned with protein sequences of YNK1, Bro1, and PalA. The asterisks indicate the identical amino acid residues, and the dots indicate similar amino acid residues. The double underlined regions indicate consensus tyrosine phosphorylation sites for the Src family kinases. The underlined sequences indicate proline-rich domains. The double underlined sequences in the underlined regions indicate PXXP motifs.

**Fig. 6.** In vitro phosphorylation of Xp95 and Xp95 peptides by Src or Fyn kinases. A, Xp95 (partially purified from oocyte extract), purified Src or Fyn kinase, or Xp95 together with either Src or Fyn kinase were incubated in the \(^{32}\)P-kinase reaction buffer at room temperature for 30 min. After samples were separated by SDS-PAGE, they were immunoblotted with anti-YNK1 antibody (left panel) and subjected to autoradiography (right panel). B, two Xp95 peptides, the 22-mer and 20-mer, were incubated with either Src or Fyn kinase in the \(^{32}\)P-kinase reaction buffer. Samples were separated by Tricine/SDS-PAGE and subjected to autoradiography.

**Fig. 7.** Phosphorylation of 22 mer and 20 mer.
cytes coincided with staining by PY99 (Fig. 7C). These results indicate that Xp95 is tyrosine-phosphorylated at low levels in oocytes and that the tyrosine phosphorylation of Xp95 increases during progesterone-induced oocyte maturation.

Finally, we examined whether increasing the level of Src kinase in oocytes affected the level of tyrosine phosphorylation on Xp95. Messenger RNA of Xenopus deregulated Src (d-Src) was produced by in vitro transcription. The RNA or H2O was microinjected into stage VI oocytes which were then incubated at 18 °C overnight to allow d-Src translation. Immunoblotting of similar amounts of proteins from H2O- or d-Src-injected oocytes as well as unfertilized eggs with anti-YNK1 antibody showed that similar amounts of Xp95 were present in different samples and that d-Src injection did not cause Xp95 gel mobility shift (Fig. 8A, left panel). Probing the same blot with PY99 showed that d-Src injection increased the amount of PY99 staining in Xp95 although the signal was still weaker than that in the Xp95 from eggs (Fig. 8A, right panel). Xp95 was then partially purified from both H2O- and d-Src-injected oocytes before being immunoblotted with anti-YNK1 and PY99. Although similar amounts of Xp95 from control and d-Src-injected oocytes were again detected by anti-YNK1 antibody, only the Xp95 band from d-Src-injected oocytes coincided with positive staining by PY99 (Fig. 8B). Finally, oocytes were injected with either Myc-tagged Xp95 RNA alone or Myc-tagged Xp95 RNA together with d-Src RNA. The Myc-tagged Xp95 protein isolated from each group of the injected oocytes by immunoprecipitation was immunoblotted with both anti-Myc antibody and PY99. Only the Myc-tagged Xp95 from the oocytes injected with both Myc-tagged Xp95 RNA and d-Src RNA coincided with staining by PY99 (Fig. 8C). These results indicate that d-Src injection increased the abundance of tyrosine-phosphorylated Xp95 and further support the possibility that Xp95 is a substrate of Src family kinases or related kinases.

**DISCUSSION**

A series of signaling events is initiated following treatment of Xenopus oocytes with the steroid hormone progesterone. These events lead to meiotic divisions and finally to the arrest of oocytes in the second meiotic metaphase. In this maturation phase of oogenesis, protein phosphorylation is known to play a crucial role in both signal transduction and meiotic induction. In this study, we observed that Xp95, a 95-kDa protein in Xenopus oocytes, undergoes a phosphorylation-dependent gel mobility shift during progesterone-induced oocyte maturation and a reverse gel mobility shift in response to fertilization. This suggests that Xp95 may be involved in the maturation process. Prompted by this possibility, we purified the protein to near homogeneity by a combination of standard chromatographic procedures and gel electrophoresis. This allowed us to obtain amino acid sequences of three internal peptides and clone a full-length cDNA encoding Xp95 by PCR-based techniques.

Analysis of the deduced amino acid sequence of Xp95 revealed that it is homologous to a conserved family of signal transduction proteins. The strongest homology was to YNK1 (51% sequence similarity), a protein of unknown function in C. elegans (24). The next was Aspergillus PaLA (37% sequence similarity), a protein that is one of six gene products implicated...
were immunoabsorbed with 1 mg of anti-Myc antibody, the immuno-

in the ambient pH regulation of gene expression (39). Xp95 was also 30% similar to mouse Rhophilin, a protein that was identified based on its association with RhoA, a member of the Ras family of monomeric G-proteins (40). RhoA has been implicated in various cellular responses to lysophosphatidic acid, tetradecanoylphorbol acetate, and certain growth factors (46). Finally, Xp95 showed 30% similarity to Bro1, a protein found in budding yeast that has been suggested by genetic studies to interact with or run in parallel with the protein kinase C-MAP kinase pathway involved in the maintenance of cell integrity (41). The homology of Xp95 to a conserved family of proteins implicated in a variety of signaling pathways suggests that Xp95 is involved in progesterone-induced signal transduction during oocyte maturation.

Alignment of the five Xp95-related proteins indicated that they fell into two subfamilies. One subfamily included Xp95, Bro1, PalA, and YNK1. These four proteins are not only homologous to each other throughout the entire length but also share three interesting features as follows: a Src kinase phosphorylation consensus sequence, six additional conserved tyrosines, and a proline-rich C-terminal domain containing multiple SH3 domain-binding motifs. The other subfamily, which thus far includes only Rhophilin, is neither homologous to the other Xp95-related proteins through the entire length nor does it contain any of the three conserved features. However, Rhophilin contains a RhoA-binding domain in its N terminus that is absent in all other Xp95-related proteins. The difference in the structure of the two subfamilies of Xp95-related proteins suggests that they transduce signals through different mechanisms.

The three conserved features of the Xp95 subfamily suggest that these proteins transduce signals, at least in part, through tyrosine phosphorylation and interaction with SH3 domain-containing proteins. Although we have not experimentally addressed the possible interaction of Xp95 with SH3 domain-containing proteins, we have obtained substantial evidence on tyrosine phosphorylation of Xp95. We demonstrated that Xp95 was phosphorylated in vitro by Src and Fyn kinases and that the phosphorylation site was likely to be in the conserved Src phosphorylation consensus sequence. In addition, we showed that Xp95 was tyrosine-phosphorylated at low levels in oocytes and that the tyrosine phosphorylation of Xp95 increased during progesterone-induced oocyte maturation. Finally, we observed that microinjection of deregulated Src message into Xenopus oocytes increased the abundance of Xp95 that is tyrosine-phosphorylated. Taken together, these results strongly suggest that Xp95 is a substrate of Src family kinases or related tyrosine kinases which participate in progesterone-induced signal transduction in Xenopus oocytes.

Besides Src family kinases or related kinases, an additional protein kinase must also phosphorylate Xp95 during progesterone-induced oocyte maturation. Xp95 undergoes a phosphorylation-independent gel mobility shift during this process. However, although the abundance of Xp95 that is tyrosine-phosphorylated increased during progesterone-induced oocyte maturation, neither the in vitro nor in vivo phosphorylation of Xp95 by Src kinase caused an obvious gel mobility shift of Xp95. This indicates that the gel mobility shift of Xp95 is caused by a different kinase that phosphorylates Xp95 on a different site(s). Since the Xp95 gel mobility shift coincided with MAP kinase activation, we have explored the possibility that this additional phosphorylation is catalyzed by MAP kinase. However, Xp95 partially purified from Xenopus oocyte extract was unable to be phosphorylated by active MAP kinase partially purified from Xenopus egg extract (data not shown). Mitotic Cdc2 kinase was also unable to phosphorylate partially purified Xp95 (data not shown). Further investigation is thus required in order to identify the kinase responsible for the Xp95 gel mobility shift during oocyte maturation.

In summary, we report here the identification and molecular cloning of a novel protein in Xenopus, Xp95, which undergoes phosphorylation during progesterone-induced Xenopus oocyte maturation. Thus far, Xp95 has three interesting features. First, it belongs to an emerging family of signal transduction proteins. Second, it is likely to be a novel substrate of Src family

FIG. 8. d-Src injection increased the abundance of tyrosine-

phosphorylated Xp95. A, 100 μg of proteins from H2O-injected oo-
cytes (Control oocytes), unfertilized eggs (Eggs), and d-Src-injected oo-
cytes (d-Src oocytes) were immunoblotted sequentially with monoclonal

antibody PY99 that recognizes phosphotyrosine and anti-YNK1 that

recognizes Xp95. B, 20 μg of partially purified Xp95 from H2O- or
d-Src-injected oocytes (total protein) were immunoblotted sequentially

with PY99 and anti-YNK1 antibody. C, after 300 μg of proteins from

doocytes injected with either Myc-tagged Xp95 RNA alone (Control oo-
cytes) or Myc-tagged Xp95 RNA together with d-Src RNA (d-Src oo-
cytes) were immunoabsorbed with 1 μg of anti-Myc antibody, the immuno-

complex was immunoblotted with anti-Myc antibody, and PY99.
kinases or related kinases. Third, it may have the capacity to bind certain SH3 domain-containing proteins. These features suggest that Xp95 is an element in a signal transduction pathway involved in progesterone-induced *Xenopus* oocyte maturation.

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