Studies on the Phosphorylation of Ser-4 in *M*, 25,000 Protein, a New Vitellogenin Gene Product Detected in *Xenopus laevis* Oocytes, Using Anti-phosphopeptide Antibody against this Site

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ABSTRACT

A phosphorylated protein with molecular mass of 25,000 (pp25), a new vitellogenin gene product [Yoshitome, S. *et al.*, unpublished results], was shown to be phosphorylated on Ser-4 by protein kinase C [Hashimoto, E. *et al.* (1996) *J. Biochem.* 119, 626-632]. In order to examine the *in vivo* phosphorylation of this site, an anti-phosphopeptide antibody was prepared and used for qualitative and quantitative analyses. This antibody specifically recognized the Ser-4-phosphorylated pp25 and did not react with Ser-4-dephosphorylated pp25. About 18% of pp25 in the cytosolic fraction was phosphorylated *in vivo*. The migration of pp25 in SDS-PAGE was increased and the proteolysis of this protein by trypsin or α -chymotrypsin was influenced by introduction of phosphate at this site. During oocyte maturation and initial stages of embryogenesis, the changes in intensity of the immunostained pp25 bands reacted with this antibody were parallel with those detected by an anti-peptide antibody recognizing all forms of pp25. These results indicate that the phosphorylation of Ser-4 actually occurs in oocytes and seems to be related to the structural change of this protein although its involvement in the functional change is not clear at this time.

A phosphorylated protein with molecular mass of 25,000 (pp25) was initially detected in our laboratory as a phosphate acceptor protein for casein kinase II in the cytosolic fraction of *Xenopus laevis* oocytes (9). In parallel with these analyses, this protein was also shown to be phosphorylated *in vitro* by protein kinase C (PK C) (9). Later, the major and minor sites phosphorylated by PK C were determined to be Ser-4 and Ser-1 in the amino-terminal region of pp25, respectively (8). Results obtained in our laboratory showed the existence of multiple endogenous phosphates (about 40-50 mol of phosphate per mol of this protein) in pp25, although the physiological role(s) of these phosphates remained to be studied (18). In an attempt to elucidate the function of

the phosphorylation of PK C site(s), the effect on protein phosphatase 2A (PP2A) activity was examined using PK C-phosphorylated and non-phosphorylated pp25 (8). Although the addition of these forms of pp25 to an *in vitro* assay system slightly stimulated the PP2A activity, the physiological significance of this phenomenon and of the phosphorylation by PK C has remained obscure (8).

It is well known that PK C plays pivotal roles in the cellular regulation of processes such as cell growth and differentiation, as described by Nishizuka (20). In *Xenopus* oocytes, the expression of subtypes of PK C was reported by Sahara *et al.* (23), and possible roles of PK C in oocyte maturation have been proposed by several investigators (26, 27). In addition, PK C may have some role(s) during embryonic development, as indicated by Otte *et al.* (21). In the present study, an anti-peptide antibody recognizing the phosphorylated Ser-4 of pp25

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was raised in a rabbit and used to survey the *in vivo* phosphorylation of this site in *Xenopus* oocytes and embryos in order to clarify the role of phosphorylation of pp25 by PK C in these systems.

MATERIALS AND METHODS

Materials and Chemicals. Frogs (*Xenopus laevis*) were mainly purchased from Johoku Seibutsu Kyozaï (Shizuoka) and maintained in an aquarium at 20°C. Wistar rats, weighing 150 to 200 g, were obtained from Shimizu Jikken Zairyo (Kyoto). BSA, trypsin, α -chymotrypsin (bovine pancreas) and progesterone were purchased from Sigma. Protein phosphatase 1 (PP1), protein phosphatase 2B (PP2B) and protein phosphatase 2C (PP2C) were purchased from Calbiochem. PP2A (dimer) and a protein phosphatase assay kit were purchased from UBI. Another protein phosphatase assay kit was also purchased from Promega. Calmodulin (bovine brain), lysyl endopeptidase, okadaic acid and citraconic anhydride were purchased from Wako (Osaka). Molecular mass markers, an enhanced chemiluminescence (ECL) kit and [γ - 32 P]ATP were purchased from Amersham Pharmacia Biotech. 2-fluoro-1-methylpyridinium toluene-4-sulfonate (FMP) Activated Cellulofine was purchased from Seikagaku Kogyo (Tokyo). Biotinylation reagents and horseradish peroxidase (HRP)-conjugated avidin were purchased from Pierce.

Preparation of Protein Fractions. *Xenopus* oocytes were prepared as described previously (28). The developmental stage of the oocytes was determined as described previously by Dumont (5). Subcellular fractions of oocytes were obtained based on the method of Blondeau and Baulieu (2) as follows: 1) oocytes were homogenized with 5 vol. of homogenizing buffer described previously (6) using a Dounce homogenizer with 20 strokes at 4°C; 2) the homogenate was centrifuged as described by Blondeau and Baulieu (2) using a low speed centrifuge (Model CR 15D, Hitachi) and a mini-ultracentrifuge (Model CS-120, Hitachi) for obtaining soluble and particulate fractions, except that the centrifugation at 10,000 $\times$ g was omitted. The soluble and the solubilized particulate fractions were heat-treated for 5 min in a boiling water bath and the heat-stable supernatant, obtained after centrifugation at 10,000 $\times$ g for 10 min, was subjected to SDS-PAGE and immunoblot analysis as indicated in various experiments.

pp25 was prepared from the heat-treated soluble

fraction of *Xenopus* oocytes through the purification step of gel filtration as described previously (7) except that the homogenizing buffer was modified as follows: 1) the concentrations of Hepes buffer, EGTA and leupeptin were changed to 20 mM, 20 mM and 7 μ M, respectively; 2) 4.4 μ M pepstatin A, 5 μ M antipain, 5 μ g/mL aprotinin, 20 mM NaF and 80 mM β -glycerophosphate were further added; 3) 60 mM β -glycerophosphate was included in the elution buffer of the anion-exchange chromatography to prevent dephosphorylation (Method I). In some experiments, oocyte homogenization was performed as indicated above in the absence of the phosphatase inhibitors to permit the dephosphorylation of pp25 by endogenous protein phosphatase(s) (Method II). In another experiment, 3.7 g (wet weight) of *Xenopus* ovaries were homogenized as described above with 5 vol of 20 mM Tris-HCl at pH 7.5 and incubated for 90 min at 37°C to permit dephosphorylation of pp25 by endogenous protein phosphatase(s), and then this mixture was heat-treated in a boiling water bath for 10 min. The supernatant obtained by centrifugation at 10,000 $\times$ g for 10 min was used as the starting material for the preparation of dephosphorylated pp25 (Method III). The same amount of *Xenopus* ovaries was heated directly in a boiling water bath for 10 min and then homogenized as indicated above. The heat-stable fraction was used as the starting material for preparing endogenously phosphorylated pp25 (Method IV). All of the pp25 preparations were purified through the gel filtration step as indicated above.

A Ni $^{2+}$ -binding protein which possesses serpin activity (*pNiXa*) was purified from *Xenopus* oocytes as described previously by Beck *et al.* (1).

Preparation of Enzymes. PK C was prepared as described earlier by Sekiguchi *et al.* (24) except that rat brains stored at -80°C were employed and the purification was carried out through the step of hydrophobic chromatography. At the stage of Thr-Sepharose, the enzyme was not bound to this column and was recovered in the pass-through fraction. The reason for this is not clear. PP2A was purified as described previously by Hermann *et al.* (12) through the step of polylysine-Agarose. The final preparations of the kinase and the phosphatase with specific activities of 500 units/mg protein and 250 units/mg protein, respectively, assayed under the respective conditions indicated below were employed in this study. The animals were treated according to the rules of the committee on treating experimental animals of our medical school.

Protein Phosphorylation and Dephosphorylation. The activities of PK C and protein phosphatases were determined by the methods described previously by Sekiguchi *et al.* (24) and Hermann *et al.* (12) and the manuals provided by UBI (for PP1) and Promega (for other phosphatases) using the respective protein phosphatase assay kits. The definition of one unit of these enzymes was given in the papers and manuals cited above. The reaction mixture (50 μ L) for the phosphorylation of pp25 (prepared by Method I; 2.5 μ g of protein) by PK C (5.1 munits) was essentially the same as that of the standard reaction mixture except that 1) the concentration of magnesium acetate was increased to 15 mM; 2) BSA was added at a final concentration of 40 μ g/mL; 3) radioactive and non-radioactive 40 μ M ATP were used for 32 P incorporation experiments and other experiments, respectively. The PK C-phosphorylated pp25 was also prepared for the dephosphorylation reaction by protein phosphatase. For this, the dephosphorylated pp25 (prepared by Method III; 5 μ g of protein) was phosphorylated by PK C for 90 min at 30°C under the conditions indicated above and stored at -20°C overnight. After defrosting, the phosphorylated pp25 from 16 reaction tubes were recovered by centrifugation at $10,000 \times g$ for 10 min and then the precipitate was dissolved in 25 mM EDTA containing 1 M NaCl and dialyzed against 20 mM Tris-HCl at pH 7.5. The reaction mixture (50 μ L) for the dephosphorylation of phosphorylated pp25 (1–2.5 μ g of protein which contained 0.54–1.6 mol of phosphate incorporated per mol of this protein) by PP2A (2 munits) purified in our laboratory or by commercially available protein phosphatases (5 munits each) contained 50 mM Tris-HCl at pH 7.5, 40 μ M CaCl₂, 1 mM NiCl₂, 125 μ g/mL BSA and other activators (10 μ g/mL calmodulin for PP2B and 60 mM magnesium acetate for PP2C) according to the assay manual indicated above or the method described by Hermann *et al.* (12). At the times indicated in both phosphorylation and dephosphorylation reactions, an aliquot (20 μ L) of the non-radioactive reaction mixture was removed and the reaction was stopped by the addition of 5-times concentrated SDS-PAGE sample buffer and heat-treatment in a boiling water bath for 3 min. The mixture was subjected to SDS-PAGE and Western blot analysis. In other experiments, an aliquot of the radioactive reaction mixture was added to an Eppendorf tube containing 1 mL of 10% (W/V) trichloroacetic acid (TCA). After the addition of 50 μ g of BSA as a carrier protein, the tube was incubated for 10 min at 80°C and acid-precipi-

tated materials were collected on a membrane filter. After washing four times with 1 mL of 10% (W/V) TCA each time, the radioactivity was measured as indicated below. pp25 was also dephosphorylated by acid phosphatase as indicated earlier (18).

Proteolysis of pp25. The Ser-4 phosphorylated (by PK C using the method for preparation of phosphatase substrate) and the Ser-4 dephosphorylated (by endogenous phosphatase(s) using Method III) pp25 (2.75 μ g each) prepared as indicated above were digested with lysyl endopeptidase (1 : 500 molar ratio) or trypsin (1 : 1000 molar ratio) or α -chymotrypsin (1 : 100 molar ratio) in a reaction mixture (110 μ L) containing 50 mM Tris-HCl at pH 8.8. At the indicated times, an aliquot of the mixture (20 μ L) was removed and 5-times concentrated SDS-PAGE sample buffer (5 μ L) was added. After heat-treatment in a boiling bath for 3 min, the samples were subjected to SDS-PAGE. Before staining with Coomassie Brilliant Blue (CBB), the gel was treated for 10 min with a solution containing 10 mM FeCl₃, 40% ethanol and 10% acetic acid to strengthen the staining of the phosphate-containing protein with the dye.

Binding of pNiXa. Biotinylated *pNiXa* was prepared as follows. Purified *pNiXa* (2 nmol) was incubated with 100 nmol of citraconic anhydride in a reaction mixture (1 mL) containing 30 mM sodium carbonate buffer at pH 8.5 at 30°C for 2 h. This treatment with citraconic anhydride was performed to minimize the binding of biotin to *pNiXa*. To this reaction mixture, 40 nmol of biotinylation reagent was added, and the mixture was further incubated as indicated above. The biotinylated *pNiXa* was dialyzed against 1 mM Tris-HCl at pH 7.5 at room temperature for 2 h, and against 50 mM acetic acid at room temperature for 2 h and at 40°C for 4 h. This preparation was finally dialyzed against phosphate-buffered saline (PBS) containing 0.05% Tween 20 (TPBS) at room temperature for 2 h and then against fresh TPBS overnight, and stored at -20°C.

After blotting as described below, the blocked membrane was incubated with 200 pmol/mL biotinylated *pNiXa*, and then with HRP-conjugated avidin at room temperature for 1 h each. *pNiXa*-binding proteins were visualized using an ECL kit.

Preparation of Antibodies. Affinity columns containing antigen peptides were prepared as follows. Swollen FMP Activated Cellulofine (3 mL) was mixed with 1 mg of peptide (the amino-terminal peptide of

pp25 SRVSKRQQEKNK DTHGC or the phosphopeptide CSRVS(P)KRQQEK, both synthesized by Sawady Technology (Tokyo)) dissolved in 50 mM sodium carbonate buffer at pH 8.5 with agitation overnight at 4°C. After the gel was blocked with 50 mM Tris-HCl at pH 8.0 containing 100 mM 2-aminoethanol, it was shaken in the presence of the latter buffer at room temperature for 3 h. The gel was then washed sequentially with water, 100 mM glycine-HCl at pH 2.5, water and 20 mM Tris-HCl at pH 7.5 containing 1 M NaCl and 1% Triton X-100 (washing buffer), and finally equilibrated with 20 mM Tris-HCl at pH 7.5 containing 150 mM NaCl.

Rabbit antisera against the two peptides obtained from Sawady Technology were purified in our laboratory on the affinity columns described above as follows. The equilibrated Cellulofine gel (about 5 mL) was mixed with the prefiltered antiserum (1.8 mL) with agitation overnight at 4°C and washed sequentially with Tris-buffered saline (TBS) (10 mL), the washing buffer described above (30 mL), TBS (30 mL) and 150 mM NaCl (10 mL), and finally the antibody was eluted with 6 mL of 100 mM glycine-HCl at pH 2.5. Three fractions eluted with this solution were obtained and their absorbance at 280 nm was measured. The second fraction (1.25 mL) supplemented with 0.1 mL of 0.1% BSA and the third fraction (2.7 mL) were dialyzed successively against 100 mM acetate buffer at pH 4.1 containing 130 mM NaCl for 1.5 h, PBS for 2 h and fresh PBS overnight at 4°C. After measurement of the concentration of the antibody by the dot blot method, the purified antibody was stored at -20°C in the presence of 50% glycerol and 0.5 mg/mL BSA.

Oocyte Maturation and Embryogenesis. Oocyte maturation experiment was performed using Dumont stage 5-6 oocytes (5) as described previously (6). At the time indicated, oocytes were collected and homogenized with the buffer indicated above (20 µL per oocyte) using a pellet pestle mixer (Kontes). After centrifugation at $10,000 \times g$ for 10 min, the supernatant was ultracentrifuged at $165,000 \times g$ for 90 min at 4°C, and the supernatant obtained was heat-treated in a boiling water bath for 10 min. The heat-stable fraction obtained after centrifugation at $10,000 \times g$ for 10 min was used for the subsequent analysis.

The experiment on *Xenopus* embryogenesis was also performed as indicated earlier (6). The developmental stage(s) of *Xenopus* embryos were determined by microscopic observation according to the

manual published previously by Nieukoop and Faber (19). Samples were taken at the indicated stages and homogenized and the heat-treated soluble fraction was prepared as described above.

Electrophoresis and Western Blotting. SDS-PAGE was performed as described by Laemmli (16) utilizing a 12.5% or 15% acrylamide running gel and a 4.5% stacking gel. Proteins in the gel were stained with CBB or transferred to a PVDF membrane using a Trans-blot SD Semi-dry Transfer Cell (Bio-Rad) or a Semi Dry Electrobloetter (Sartorius) at 6 V for 2 h as described previously (10). The procedures for blocking with 5% skim milk solution and the subsequent reactions with antibodies were essentially the same as those described earlier (10). The antibody was diluted 500-1,000 times for use as the primary antibody. In all Western blot experiments, an ECL kit was employed to detect immunostained protein(s) except in the experiment shown in Fig. 1, where 3,3'-diaminobenzidine was employed for color development. All of the protein bands with M_r 25,000 disappeared when the primary antibody was premixed with a large amount of the antigen (data not shown).

Other Procedures and Determinations. Protein was determined by the method of Bradford (3) with BSA as a standard. The concentration of pp25 in stained SDS-PAGE gels was determined as described previously (9). The relative amount of pp25 on films after immunostaining and development using an ECL kit was also determined by densitometric analysis as described above or by using NIH Image (version 1.62) after the data were recorded using a scanner. Radioactivity was determined with a Beckman LS-5801 liquid scintillation counter by measuring Cerenkov radiation.

RESULTS

Detection of Ser-4 Phosphorylation by Phosphopeptide Antibody

In a previous report from our laboratory we identified Ser-4 in pp25 as the major site of phosphorylation by PK C (8). Using a phosphopeptide antigen with the amino acid sequence around Ser-4, a phosphopeptide antibody was raised in white rabbits. Initially, the basic properties of this antibody were examined using pp25 purified in the presence or absence of protein phosphatase inhibitors. The results shown in Fig. 1 indicate that the antibody reacted with pp25 purified in the presence of the phos-

Fig. 1 Detection of Ser-4 phosphorylation in pp25 by phosphopeptide antibody. The cytosolic fractions of *Xenopus* oocytes were prepared according to Methods I and II. The protein concentration was adjusted to 2.5 mg/mL and an aliquot of each sample was heat-treated. An aliquot of each supernatant (20 μ L) was separated by SDS-PAGE and subjected to Western blot analysis using the phosphopeptide antibody. Lanes 1 and 3; samples prepared by Method I, lanes 2 and 4; samples prepared by Method II. Lanes 1 and 2; protein staining pattern by CBB, lanes 3 and 4; Western blot analysis. Numbers on the left show the positions of molecular mass markers in kDa: 94, phosphorylase *b*; 67, BSA; 43, ovalbumin; 30, carbonic anhydrase; 20, trypsin inhibitor; 14, lysozyme. The arrow on the right shows the position of pp25.

phatase inhibitors. The immunostained band was not detected when the primary antibody was omitted or the antibody was premixed with an excess of the antigen phosphopeptide (data not shown). When the inhibitors were omitted from the preparation buffer, this antibody did not react with pp25 in spite of the presence of this protein as a heat-stable protein with molecular mass of around 25,000. This result suggests that pp25 contains endogenous phosphate at Ser-4.

In order to obtain further evidence showing that this antibody recognizes the phosphorylated Ser-4 in pp25, the changes in the reactivity of the antibody were examined during the phosphorylation and dephosphorylation of pp25 *in vitro*. The results shown in Figs. 2 and 3 indicate that the intensities of the immunoreactive bands of pp25 increased or decreased gradually with time according to the changes in phosphate content (mainly in Ser-4) dur-

ing phosphorylation by PK C and dephosphorylation by PP2A, respectively. The kinase reaction proceeded in a Ca^{2+} -phospholipid-dependent manner and the phosphatase reaction was completely inhibited by okadaic acid. Judging from previously reported results (8) and the amount of phosphate incorporated or released during these reactions (from 0 to about 1.5 mol phosphate per mol of pp25), the antibody seemed to recognize the phosphorylation and dephosphorylation at Ser-4. A previous report from our laboratory indicated that pp25 was converted to a *M*, 20,000 protein (which had lost most of the endogenous phosphate) by treatment with acid phosphatase (18). However, in the present study the molecular mass of pp25 as detected by protein staining analysis was not detectably changed by PP2A treatment (data not shown). This result indicates that most of the endogenous phosphate was not removed, and instead the phosphate(s) at specific site(s) such as Ser-4 were removed by PP2A. The possibility that proteolysis occurred during phosphatase treatment was ruled out by an experiment in which protease inhibitors were included (data not shown). The results shown in Fig. 4 indicate that PP2A and PP2B can catalyze the dephosphorylation of this PK C site effectively, but PP1 and PP2C are ineffective.

Next, the content of endogenous phosphate at Ser-4 was estimated. For this analysis, the endogenous phosphate remaining at Ser-4 in pp25 prepared in the absence of phosphatase inhibitors was removed by the action of endogenous protein phosphatase(s). This preparation did not react with the anti-phosphopeptide antibody in Western blot analysis (data not shown) and served as a phosphate acceptor for PK C. After incorporation of one mol of phosphate per mol of pp25, various amounts of the phosphorylated pp25 were subjected to Western blot analysis and visualized with the antibody as shown in Fig. 5A. By scanning these protein bands, a standard curve of the relative area of Ser-4-phosphorylated pp25 versus the amount of this protein was made (Fig. 5B). Using this curve, the endogenous phosphate content of pp25 prepared in the presence of phosphatase inhibitors was calculated to be 0.18 mol of phosphate per mol of this protein. This result indicates that about one fifth of pp25 is phosphorylated at Ser-4 in *Xenopus* oocytes.

Effect of Ser-4 Phosphorylation on Properties of pp25

Next we attempted to elucidate the specific role of Ser-4 phosphorylation in multiply phosphorylated

Fig. 2 Immunoblot analysis of pp25 during phosphorylation by PK C. Experiment A, time course of phosphorylation of pp25. pp25 (prepared by Method I) was phosphorylated by PK C and the incorporated radioactivity was determined under the conditions described in 'MATERIALS AND METHODS' except that the reaction conditions were modified as indicated below. (○), complete system; (△), minus Ca²⁺, phosphatidylserine and diolein; (□), minus pp25. Experiment B, Western blot analysis using phosphopeptide antibody. At the indicated times, samples were taken from the complete system in six separating tubes for immunoblot analysis. Arrows on the right show the position of pp25 (bottom) and an unidentified protein (top). Other conditions are the same as described in the legend to Fig. 1. Lanes 1–6 show samples taken at 0, 2.5, 5, 10, 30 and 60 min, respectively.

Fig. 3 Immunoblot analysis of PK C-phosphorylated pp25 during dephosphorylation by PP2A. Experiment A, time course of dephosphorylation of PK C-phosphorylated pp25. The preparation of ³²P-labelled pp25 (2.5 µg per assay tube, 1.6 mol phosphate per mol of pp25) and the phosphatase assay by PP2A (purified in our laboratory) were performed under the conditions described in 'MATERIALS AND METHODS' except that the reaction mixture was 125 µL and the reaction conditions were modified as indicated below. (○), complete system; (△), minus PP2A; (□), plus okadaic acid (100 nM). Experiment B, Western blot analysis using phosphopeptide antibody. At the indicated times, samples were taken from the complete system for immunoblot analysis as indicated in the legend to Fig. 2. Arrows on the right show the position of pp25 (bottom) and an unidentified protein (top). Other conditions were the same as described in the legend to Fig. 1. Lanes 1–5 show samples taken at 0, 5, 10, 30 and 60 min, respectively.

Fig. 4 Dephosphorylation of PK C-phosphorylated pp25 by various protein Ser/Thr phosphatases. The preparation of ^{32}P -labelled pp25 (1 μg per assay tube, 0.54 mol phosphate per mol of pp25) and the phosphatase assay by various protein Ser/Thr phosphatases were performed under the conditions described in 'MATERIALS AND METHODS' except that the reaction mixture was 100 μL and the result at each point was obtained using the reaction mixture from one tube. Other conditions were the same as those indicated in the legend to Fig. 3. (○), with PP1; (△), with PP2A (the dimer commercially-available); (□), with PP2B; (×), with PP2C.

pp25. In earlier studies, pp25 was detected as a diffuse band by protein staining after gel electrophoresis (9). We supposed that this phenomenon might be due to variation of the level of endogenous phosphorylation. In recent experiments, this diffuse protein band was detected several times as separated protein bands. As shown in Fig. 6A, the protein-staining band shifted from the usual position of *M.* 25,000 to a faster migrating one in parallel with the phosphorylation by PK C. In the absence of ATP, the position of this band did not change. The same analysis was performed using Western blot analysis. When the phosphopeptide antibody was employed, it reacted mainly with the faster migrating band (Fig. 6B). pp25 dephosphorylated by endogenous protein phosphatase(s) did not react with this antibody at time 0, and pp25 incubated with PK C in the absence of ATP also did not react with it. In contrast, the slower migrating pp25 was mainly detected at time 0 using the amino-terminal peptide antibody, and the intensity of the faster migrating band increased in parallel with the progression of the phosphorylation reaction (Fig. 6C). In addition, this mobility shift

during phosphorylation was observed in the presence of protease inhibitors (data not shown). The slight difference in the position of pp25 immunostained with the phosphopeptide antibody during phosphorylation by PK C seems to be mainly due to the structural change by incorporation of phosphate into Ser-4.

In the next experiment, the binding of *pNiXa* to various forms of pp25 that differed with respect to Ser-4 phosphorylation was examined. Previous results from this laboratory indicated that *pNiXa* is the major binding protein for pp25 in the cytosolic fraction of *Xenopus* oocytes (7). In the previous overlay assay, the binding proteins on PVDF membranes were surveyed using ^{32}P -labelled pp25 as a probe (7). In the present study, four types of PVDF-membrane-bound pp25 prepared with different phosphorylation conditions were examined for their ability to bind *pNiXa*, because it was easier to prepare just one probe. To avoid multiple levels of biotinylation, the *pNiXa* was initially modified by citraconic anhydride and then biotinylated. After this modification, most of the citraconic acid on lysyl residues was removed by acid treatment. The results shown in Fig. 7 indicate that all of the phosphorylated forms of pp25 (pre-treated with partially purified PP2A to remove mainly endogenous phosphate at Ser-4, lane 2; prepared in the presence of phosphatase inhibitors to keep endogenous phosphate at Ser-4, lane 3; phosphorylated by PK C up to about one mol of phosphate per mol of pp25 protein, lane 4) could bind biotinylated *pNiXa*, but the dephosphorylated form of pp25 (which had been pre-treated with potato acid phosphatase and had lost most of its endogenous phosphate (18), lane 1) could not bind it. Previous analyses showed that the phosphate residue(s) on pp25 are important for the binding of pp25 to the histidine-rich domain of *pNiXa* (7). Although some role of the phosphorylation of Ser-4 of pp25 is therefore expected, the effect of this phosphorylation on the inhibitory activity of *pNiXa* toward serine protease was not examined in this study based because we detected no difference between the binding of Ser-4-phosphorylated and -dephosphorylated pp25 to *pNiXa*.

Previous reports from other laboratories showed that the susceptibility of protein to proteolytic attack was influenced by the phosphorylation by PK C (17, 22). Therefore, the susceptibilities of Ser-4-phosphorylated and -dephosphorylated pp25 to three proteases with different substrate specificities were compared. The protein staining patterns of the fragments generated by digestion with lysyl endopepti-

Fig. 5 Estimation of endogenous phosphate at Ser-4 in cytosolic pp25. The pp25 dephosphorylated by endogenous phosphatase(s) and that containing endogenous phosphate were prepared by Methods III and IV, respectively. The dephosphorylated pp25 was phosphorylated by PK C under the conditions indicated in 'MATERIALS AND METHODS'. This non-radioactive pp25 contained 1 mol of newly incorporated phosphate per mol of this protein judging from the radioactivity in the parallel reaction sample. Most of this phosphate was assumed to be incorporated in Ser-4 [8]. Various amounts of this phosphorylated pp25 (0.025–0.4 µg) were subjected to SDS-PAGE and Western blotting was performed using the phosphopeptide antibody as described in the legend to Fig. 1. Experiment A, Western blot analysis. Left six lanes and right one lane show the immunoblotting pattern of various amounts of Ser-4-phosphorylated pp25 (0.025, 0.05, 0.1, 0.2, 0.3 and 0.4 µg from lane 1 to lane 6, respectively) and 1 µg of endogenously phosphorylated pp25 (lane 7), respectively. Experiment B, Estimation of endogenous phosphate content in pp25. The area of both pp25 band (indicated by the top arrow) and the faster migrating band (indicated by the bottom arrow, the exact entity of this band is not clear at this time.) in Experiment A was calculated by NIH Image as indicated in 'MATERIALS AND METHODS' and presented by arbitrary unit. The results are plotted against the amounts of PK C-phosphorylated pp25. The area of the band of endogenously phosphorylated pp25 was the average of two analyses. (○), PK C-phosphorylated pp25; (□), endogenously phosphorylated pp25.

Fig. 6 Mobility shift in SDS-PAGE analyses of pp25 during phosphorylation by PK C. The dephosphorylated pp25 was phosphorylated by PK C as described in 'MATERIALS AND METHODS'. At the times indicated, an aliquot (10 µL containing 1 µg of pp25) of the reaction mixture was removed and subjected to SDS-PAGE and Western blot analyses. The results of the pattern of protein staining by CBB (A), and the patterns of Western blot analyses using the phosphopeptide antibody (B) and the amino-terminal peptide antibody (C) are shown. The positions of the original pp25 (top) and PK C-phosphorylated pp25 (bottom) are indicated by arrows on the left. Lanes 1–5 show the samples taken at 0, 5, 15, 30 and 60 min, respectively. Lane 6 shows the ATP-minus control sample.

Fig. 7 Binding of *pNiXa* to pp25. Acid phosphatase-treated pp25 (prepared by Method I), PP2A (purified in our laboratory)-treated pp25 (prepared by Method I), endogenously phosphorylated pp25 (prepared by Method I) and biotinylated *pNiXa* were prepared as described in 'MATERIALS AND METHODS'. PK C-phosphorylated pp25 was prepared as described in the legend to Fig. 5. One-microgram aliquots of pp25 were subjected to SDS-PAGE and stained with CBB or transferred to a PVDF membrane, and then the binding of biotinylated *pNiXa* was carried out as described in 'MATERIALS AND METHODS'. The results of protein staining (A) and *pNiXa* binding (B) are shown. The positions of pp25 (top), PK C-phosphorylated pp25 (middle) and acid phosphatase-treated pp25 (bottom) are shown by arrows on the right. Lane 1, acid phosphatase-treated pp25; lane 2, PP2A-treated pp25; lane 3, endogenously phosphorylated pp25; lane 4, PK-C-phosphorylated pp25. Other conditions are the same as those described in the legend to Fig. 1. The heavily stained bands in lanes 2 and 4 around molecular mass of 67,000 in Experiment A are BSA added as a stabilizer for PP2A and PK C, respectively.

dase did not seem to be different for these two preparations of pp25 (data not shown). But in the cases of trypsin and α -chymotrypsin, the patterns were slightly different and unique protein bands were detected for Ser-4-phosphorylated and -dephosphorylated pp25 as shown in Fig. 8. Although the rate of disappearance of each original form seemed to be essentially the same for these two preparations, Ser-4-phosphorylation affected some protease action presumably due to the structural change of pp25.

Changes in Ser-4 Phosphorylation in Biological Systems

The results shown in Fig. 1 suggest that Ser-4 in

Fig. 8 Comparison between protease-degradation patterns of PK C-phosphorylated and dephosphorylated pp25. The preparation of the phosphorylated and dephosphorylated forms of pp25 (both forms were prepared by Method III and the former was further phosphorylated by PK C) and their digestion by three proteases were performed as described in 'MATERIALS AND METHODS'. The protein-staining patterns after gel electrophoresis are shown. The left and right sets of five lanes each are derived from the phosphorylated and dephosphorylated pp25, respectively. (A), with trypsin; (B), with α -chymotrypsin. Lanes 1-5 show the samples taken at 0, 2.5, 5, 7.5 and 10 min, respectively. An arrowhead and arrows on the right in Experiments A and B show the position of the specific bands detected using the PK C-phosphorylated and the dephosphorylated pp25. Other conditions are the same as those described in the legend to Fig. 1.

pp25 is phosphorylated in resting *Xenopus* oocytes. Based on these results, further analyses were performed on samples obtained from oocytes under various physiological conditions. During the course of oocyte maturation induced by progesterone (Fig. 9A), there was no change in the immunoblotting pat-

tern of pp25 obtained using the phosphopeptide antibody, as shown in Fig. 9B. Consistent with the previously reported results using pp25 whole protein antibody (6), the pattern obtained using the amino-terminal peptide antibody also showed no appreciable change during oocyte maturation.

In a previous report, pp25 was shown to be degraded at Nieukoop/Faber stages 40–45 during embryogenesis (6). In the last experiment, the changes in pp25 during embryogenesis were also examined by immunoblotting using the anti-phosphopeptide and anti-amino-terminal peptide antibodies. The results shown in Figs. 10 indicate that the pp25 bands detected by both antibodies disappeared after Nieukoop/Faber stage 40. Judging from these data, no clear difference was observed between these immunostained pp25 bands detected by the respective antibodies throughout the time course of the development. The amount of immunostained band of pp25 in the particulate fraction was negligible compared with that in the cytosolic fraction and was not analyzed further (data not shown).

DISCUSSION

In this study, the phosphorylation of Ser-4 in pp25 was suggested to occur in resting oocytes based on immunoblotting using the phosphopeptide antibody. The phosphate incorporation at Ser-4 may play some role in its function. Although the ability to bind to *pNixA* was not clearly affected, the susceptibility to trypsin and α -chymotrypsin was modified by Ser-4 phosphorylation. In addition, the mobility in SDS-PAGE became faster by Ser-4 phosphorylation. These results suggest that the Ser-4 phosphorylation may be involved in the structural change of pp25 protein.

It has been suggested by several investigators that PK C might play important roles during oocyte maturation and embryogenesis (21, 25–27). Judging from the amino acid sequence around Ser-4 of pp25, PK C seems to be the most probable candidate as the enzyme catalyzing the phosphorylation of this site, as pointed out by Kennelly and Krebs (15). As the Ser-4 of pp25 was phosphorylated effectively by

Fig. 9 Immunoblot analyses of pp25 using peptide antibodies during oocyte maturation. Experiment A; time course of oocyte maturation induced by progesterone. Induction of maturation was performed as described in 'MATERIALS AND METHODS'. (○), with progesterone; (□), with vehicle. Experiments B and C; immunoblot analyses. At the times indicated, 25 oocytes were collected and the heat-stable cytosolic fraction was prepared using the homogenizing buffer for immunoblot analysis using anti-peptide antibodies under the conditions described in the legend to Fig. 1. The results using progesterone-treated (right 5 lanes) and vehicle-treated (left 5 lanes) samples are presented. Samples corresponding to 0.5 oocyte per well were analyzed. Arrows on the right show the position of pp25. (B), with the amino-terminal peptide antibody; (C), with the phosphopeptide antibody. Lanes 1–5 show samples taken at 0, 2, 4, 6 and 8 h, respectively. Other conditions are the same as those described in the legend to Fig. 1.

Fig. 10 Immunoblot analyses of pp25 using anti-peptide antibodies during *Xenopus* embryogenesis. Experiments A and B; immunoblot analyses. At the times indicated, 10 embryos were collected and the heat-stable cytosolic fraction was prepared using the buffer for immunoblot analysis. Immunoblots were stained with the anti-peptide antibodies under the conditions described in the legend to Fig. 9. The results using the amino-terminal peptide antibody (A) and the phosphopeptide antibody (B) are presented. Arrows on the right show the position of pp25. Lanes from left to right show the samples obtained from unfertilized eggs and fertilized eggs at Nieuwkoop/Faber stages 1, 5, 10, 15, 20, 25, 30, 35, 40 and 45, respectively (0.2 embryo/well). Other conditions are the same as those described in the legend to Fig. 1.

PK C in an *in vitro* reaction system (8), it seems to be the catalyst participating in the phosphorylation of Ser-4 in pp25 *in vivo*.

In the previous study from our laboratory, the binding protein of pp25 was surveyed in order to get some clue on the role of this protein (7). As the result, *pNiXa*, a kind of serpin reported by Beck *et al.* (1), was identified as the binding protein detected in

the cytosolic fraction (7). This protein was also known as Ep45 whose mRNA synthesis was enhanced in *Xenopus* liver by estrogen treatment as described by Holland *et al.* (13). For the binding with pp25, the interaction between the histidine-rich domain of *pNiXa* on the amino-terminal region and the phosphorylated residue(s) of pp25 was shown to be important (7). Previous report by Dela Cadena and Colman indicated that the histidine-rich domain in the light chain of high molecular weight kininogen serves as a primary structural feature for zinc-dependent binding to an anionic surface (4). This mechanism may be applied to the binding of pp25 with *pNiXa* because this interaction was enhanced in the presence of zinc in the overlay assay system (Sugimoto, I. *et al.*, unpublished results).

More recently, cDNA cloning of pp25 was performed in our laboratory and the result shows that pp25 is one of the components of a new vitellogenin gene product and seems to be a cytosolic yolk protein (Yoshitome, S. *et al.*, unpublished results). The result of cDNA cloning indicates the existence of two serine-rich domains (many of these residues seem to be phosphorylated judging from the high phosphate content of pp25 reported previously (18)) separating each other in pp25 molecule. Although this is a speculation, pp25 may be a kind of adaptor protein possessing two binding sites of multiply phosphorylated serine residues toward a group of target proteins containing histidine- or basic amino acid-rich domain. The *pNiXa* was shown to be involved in oocyte maturation by influencing *ras* function and in normalization of abnormal *Xenopus* embryogenesis induced by mRNA injection of follistatin as reported by Haspel *et al.* (11) and Iemura *et al.* (14), respectively. Based on these facts, the binding protein(s) for the pp25-*pNiXa* complex is being surveyed in our laboratory in parallel with the identification of the binding proteins of pp25 detected in the particulate fraction of oocyte (1% Triton X-100-insoluble fraction) (7). After determining the profile of binding proteins of pp25, the role of phosphorylation of Ser-4 will be re-examined from the points of its effect for binding these proteins or modulating the functions of these proteins. As reported recently by Funami *et al.*, pp25 exists in resting and maturing oocytes and eggs at the initial stage of embryogenesis (till around Nieuwkoop/ Faber stage 40) (6). After that, both pp25 and *pNiXa* disappeared in parallel (6). These results suggest that pp25 may play some important role during oocyte maturation and initial stage of embryogenesis.

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