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A Serpin with M_r 43,000 Is a Binding Protein of M_r 25,000 Protein, a Substrate for Protein Ser/Thr Kinase Detected in *Xenopus laevis* Oocytes[1]

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In an attempt to get some clue as to the function of M_r 25,000 protein, a protein Ser/Thr kinase substrate detected in *Xenopus laevis* oocytes [Hashimoto, E. *et al.* (1995) *J. Biochem.* 118, 453–460], the binding protein was surveyed using the ^{32}P-labeled protein by casein kinase II as a screening probe. When the cytosolic proteins from oocytes were transferred to a polyvinylidene fluoride membrane and incubated with the labeled protein, only one protein with M_r 43,000 was visualized on autoradiography. This protein was purified to a nearly homogeneous state through several column chromatography steps. The amino acid sequence of the amino-terminal region of this protein identified it as a kind of serine protease inhibitor (serpin) [Holland, L.J. *et al.* (1992) *J. Biol. Chem.* 267, 7053–7059]. However, the M_r 25,000 protein did not have any effect on the inhibitory action of this serpin on α-chymotrypsin. In addition, several binding proteins were also detected in the particulate fraction of oocytes, although the exact identity of these proteins is not clear at this time. These results suggest that the M_r 25,000 protein may play some role(s) by interacting with these binding proteins in *Xenopus* oocytes.

Key words: binding protein, overlay assay, protein Ser/Thr phosphorylation, serine protease inhibitor (serpin), *Xenopus laevis* oocytes.

In a previous report from this laboratory, we described the purification of a M_r 25,000 protein (pp25) from the cytosolic fraction of *Xenopus laevis* oocytes as a phosphate acceptor protein for CK II and PK C (*1*). This protein is heat-stable and contains multiple endogenous phosphates that can be removed by treatment with acid phosphatase (*2*). Judging from the amino acid sequence of the amino-terminal portion of pp25, this protein seemed to be a newly identified protein (*1*). In an attempt to elucidate the function of pp25, we first examined the probability of a modulator protein of PP2A based on the facts that this protein is detected mainly in the cytosolic fraction of resting oocytes (*1*) and that PP2A has been shown to be a negative regulator of cell cycle G_2/M transfer (*3*). Although the highly purified preparation of pp25 slightly stimulated the *p*-nitrophenyl phosphatase activity of PP2A, the significance of this phenomenon was not clear because a high concentration of pp25 was necessary despite the weak stimulation (*4*). At present, the physiological role of pp25 is not well established. In order to obtain some clue as to the role of pp25 in oocytes, we analyzed the binding protein(s) of this protein using an overlay assay procedure. As a result, a M_r 43,000 protein (p43) detected in the cytosolic fraction was identified as Ep45 (a kind of serpin) (*5*) or *pNiXa* (*6*).

EXPERIMENTAL PROCEDURES

Materials and Chemicals—Xenopus laevis females were purchased from Sanai Shoji (Saitama), Copacetic (Aomori) or Johoku Seibutsu Kyozai (Shizuoka) and maintained in aquariums at 25°C. *Xenopus* oocytes were prepared as described previously (*1*). For subcellular fractionation, oocytes were homogenized at 4°C by 20 strokes of a Dounce homogenizer in 5 times homogenizing buffer containing 10 mM HEPES, pH 7.6, 83 mM NaCl, 1 mM $MgCl_2$, 10 mM EGTA, 1 mM PMSF, and 47 μM leupeptin. The homogenate was centrifuged at 100 ×*g* for 10 min and the resultant supernatant was further centrifuged at 165,000 ×*g* for 90 min. The supernatant was used as the cytosolic fraction and the precipitate was re-homogenized in the same buffer containing 1% Triton X-100. The solution was ultracentrifuged

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Abbreviations: CK II, casein kinase II; ECL, enhanced chemiluminescence; Ep45, estrogen-induced protein with a molecular mass of 45,000; PK C, Ca^{2+}-phospholipid-dependent protein kinase; *pNiXa*, a Ni^{2+}-binding protein in *Xenopus* oocytes and embryos; p43, protein with a molecular mass of 43,000; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; pp25, phosphorylated protein with a molecular mass of 25,000; PP2A, protein phosphatase 2A; PTH, phenylthiohydantoin; PVDF, polyvinylidene fluoride; serpin, serine protease inhibitor; TPBS, phosphate-buffered saline containing 0.05% Tween 20.

again, and the supernatant and precipitate were used as solubilized and non-solubilized particulate fractions, respectively. BSA, α-casein, trypsin (bovine pancreas), α-chymotrypsin (bovine pancreas), acid phosphatase (white potato), actin (rabbit skeletal muscle), 3,4-dichloroisocoumarin, and *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide were obtained from Sigma. DEAE-Sephacel, Sephacryl S-200, HiTrap Chelating, molecular mass markers for SDS-PAGE, an ECL kit, and [γ-^{32}P]ATP were purchased from Amersham Pharmasia Biotech. Affi-gel 15 and phosphocellulose were obtained from Bio-Rad and Whatman, respectively. Leupeptin, pepstatin, and antipain were purchased from Peptide Institute. A Silver Staining Kit II and estradiol-17β were from Wako. Aprotinin was from Nakalai Tesque. PVDF membranes and membrane filters (pore size, 0.45 μm) were from Millipore and Advantec, respectively. ImmunoPure™-immobilized avidin and NHS-SS-biotin were from Pierce. The affinity column for pp25 was prepared using Affi-Gel 15 as indicated below. The gel (11 ml) was washed with deionized water and then equilibrated with the coupling buffer (0.1 M MOPS at pH 7.0). The gel was mixed with 5.3 mg of pp25 in the coupling buffer overnight at 4°C. After blocking with 1 M ethanolamine-HCl at pH 8.0, the column was washed with 10 volumes of coupling buffer and 3 M NaSCN, and equilibrated with PBS. Before being used for affinity column chromatography, the column was washed with 0.1 M Tris-HCl, pH 8.5, and 0.1 M acetate buffer, pH 4.5, each containing 0.5 M NaCl, and equilibrated with the starting buffer. The antibody against *pNiXa* was a kind gift from Dr. F.W. Sunderman Jr.

Purification of Proteins—pp25 was purified as described previously (*2*) from the heat-treated fraction. After DEAE-Sephacel column chromatography, the concentrated pp25 preparation was further purified by gel filtration on a Sephacryl S-100 column (1.5 × 120 cm) equilibrated with buffer containing 20 mM Tris-HCl, pH 7.5, 10 mM 2-mercaptoethanol, and 0.3 M sucrose. Fractions of 2.6 ml each were collected at a flow rate of 4.2 ml/h. The protein solution eluted in fractions 34 to 38 was concentrated to 2 ml using an Amicon ultrafiltration cell equipped with a PM-10 membrane filter. After dialysis against buffer containing 20 mM Tris-HCl, pH 7.5, 10 mM 2-mercaptoethanol, and 0.5 mM EGTA, pp25 was stored at −80°C until use. *pNiXa* was purified by the method described by Beck *et al.* (*6*) except that DEAE-Sephacel was used instead of DE-52 resin. CK II was highly purified as described previously (*1*) except that 25 g of rat brain was homogenized in 6 volumes of buffer containing 20 mM Tris-HCl, pH 7.5, 0.25 M sucrose, 10 mM EGTA, 2 mM EDTA, and 1mM PMSF, and the supernatant obtained after ultracentrifugation at 200,000 ×*g* for 30 min was employed as the starting material.

Enzyme Assays—CK II activity was measured as described earlier (*1*) except that 50 μg of BSA was added to each reaction tube as a carrier protein after stopping the reaction. One unit of enzyme was defined as the amount of enzyme that incorporates 1 nmol of phosphate per min under the above conditions. The specific activity of CK II was 340 units/mg protein. α-Chymotryptic activity was measured at 37°C under the conditions described previously (*7*) in a 1 ml reaction volume. Release of *p*-nitroaniline from the peptidyl-*p*-nitroanilide substrate was monitored at 410 nm for 140 min at intervals of 20 min using a spectrophotometer (Unidec-210, Jasco). The concentration of *p*-nitroaniline was determined using an $\varepsilon_{406\ nm}$ value of 9,920 (*8*).

Overlay Assay—For preparation of the probe, pp25 (4 μg) was phosphorylated in a reaction mixture (100 μl) containing 20 mM Tris-HCl, pH 7.5, 15 mM magnesium acetate, 0.47 mM leupeptin, 5 mM EGTA, 0.1% 2-mercaptoethanol, 0.26 mg/ml BSA, 10 μM [γ-^{32}P]ATP (3–5 × 10^6 cpm/nmol) and CK II (0.01 unit) for 90 min at 30°C. The reaction was started by the addition of ATP and stopped by heat-treatment at 80°C for 10 min. Just before stopping the reaction, 5 μl of the mixture was removed and added to a test tube containing 10% trichloroacetic acid. After the addition of 50 μg of BSA as a carrier protein, the mixture was heated for 10 min at 80°C to decompose the radioactive ATP. The radioactivity incorporated into pp25 was determined as described for the assay of CK II. About 1.0–1.7 mol phosphate was incorporated per mol of pp25. The proteins separated by SDS-PAGE were transferred to a PVDF membrane as indicated below. The membrane was blocked in TPBS containing 5% skim milk for 1 h at room temperature. The membrane was washed twice for 5 min each with TPBS and twice with binding buffer containing 10 mM sodium phosphate buffer, pH 7.4, 100 mM NaCl, 0.2 mM NaN_3, and 0.2 mM EGTA. The constituents of the overlay solution (8 ml) were as follows: 95 μl of the ^{32}P-labeled pp25 described above, 2.5 μl of non-labeled pp25 (50 μg), and 7.9 ml of binding buffer. The membrane was then incubated with this overlay solution (2.6 ml) in the presence of an additional 88 μl of the pp25 buffer or the same volume of protein (1.76 mg) overnight at 4°C with gentle shaking. Finally the membrane was washed 3 times with binding buffer, air dried, and subjected to autoradiography with an intensifying screen for 12 h at −80°C. As another control experiment, overlay solution containing only non-labeled pp25 (to which heat-denatured CK-II was added) was employed to exclude the possibility of [γ-^{32}P]ATP binding.

The probe using the dephosphorylated pp25 was prepared as indicated below. The purified pp25 (0.5 mg) was dephosphorylated in a reaction mixture (1 ml) containing 30 mM MES, pH 6, 3 mM EGTA and 1 μg/ml acid phosphatase for 90 min at 30°C. The reaction was stopped by heat-treatment at 80°C for 10 min. The dephosphorylated and phosphorylated pp25 incubated in the absence of acid phosphatase (0.16 nmol each) was phosphorylated with CK II (5.9 milliunits) in the reaction mixture indicated above for 60 min at 30°C. Under these conditions, 3.8 and 1.5 mol phosphate were incorporated per mol of dephosphorylated and phosphorylated pp25, respectively. The radioactivity per mol of each protein was adjusted to be same by the addition of dephosphorylated pp25 labeled with non-radioactive ATP. For overlay assay, 1.5 μg of *pNiXa* per lane was subjected to SDS-PAGE in a 10% acrylamide running gel and transferred to a PVDF membrane as indicated below. After blocking, the membrane was washed as indicated above. The membrane strips were incubated overnight with 3 ml of a solution containing the dephosphorylated pp25 labeled with radioactive ATP (95 μl containing 0.16 pmol protein, 2.3 × 10^5 cpm), dephosphorylated pp25 (32 μl containing 0.64 pmol protein) and binding buffer (2.87 ml) or the same volume of solution containing the radioactive pp25 and non-labeled pp25 at the amounts indicated above. After washing with binding buffer as indicated above, the

strips were dried and subjected to autoradiography for 24 h.

Overlay assay of *pNiXa* subjected to limited proteolysis with trypsin was performed as indicated below. *pNiXa* (100 pmol) was digested with trypsin (12 pmol) at 23.5°C in the reaction mixture (50 μl) described previously (*7*). The reaction was stopped at 0 min, 10 min or 30 min by the addition of 5 μl of 10 mM 3,4-dichloroisocoumarin. After the addition of 12.5 μl of 5 times concentrated SDS-PAGE sample buffer, the samples were boiled for 3 min and 20 μl of each sample was subjected to SDS-PAGE (10% acrylamide running gel) and Western blotting as indicated below. A set of three lanes was used for the usual overlay assay using ^{32}P-labeled pp25 as indicated above.

Biotinylation of pp25 and Binding to p43—pp25 was biotinylated using ImmunoPure™ NHS-SS-biotin according to the manufacturer's instructions. In brief, 40 nmol of pp25 (in 1 ml of 50 mM $NaHCO_3$, pH 8.5, containing 0.02% Triton X-100) was mixed with 400 nmol of NHS-SS-biotin (in 25 μl of the same buffer) and the reaction was allowed to proceed for 3 h at 25°C. After the addition of another 400 nmol of NHS-SS-biotin, the reaction was continued for an additional 16 h. Biotinylated pp25 was separated from the non-reactive reagent by HPLC using a TSKgel QAE-2SW (0.46 × 25 cm, TOSOH) eluted with a linear gradient of NH_4HCO_3 from 50 to 500 mM. The modified pp25 was used directly in the next assay. The binding ability of the biotinylated pp25 to p43 was confirmed by the overlay assay indicated above. For the binding experiment, the cytosolic fraction of *Xenopus* oocytes (100 μl, about 100 μg of protein) dialyzed against the binding buffer was mixed first with the immobilized-avidin (30 μl) in the presence of 0.1% Triton X-100 for 1 h at 4°C with rotation. After centrifugation at 5,000 ×*g* for 3 min, the supernatant was mixed with the modified pp25 (16 μl, 2 μg of protein) or pp25 buffer for 1 h, and then in the presence of immobilized-avidin (10 μl) for 1 h as indicated above. The mixture was centrifuged at 5,000 ×*g* for 3 min, and the precipitate was washed 4 times with the binding buffer containing 0.1% Triton X-100 and suspended in 50 μl of distilled water. An aliquot was dissolved in SDS-PAGE sample buffer and boiled for 3 min. After brief centrifugation, the supernatant was subjected to SDS-PAGE and Western blot analysis as described below.

Electrophoresis and Western Blotting—SDS-PAGE was performed as described previously (*9*) utilizing a 12.5% acrylamide running gel and a 4.5% stacking gel. Proteins on the gel were transferred to a PVDF membrane using a Trans-blot SD Semi-dry Transfer Cell (Bio-Rad) at 12 V for 40 min according to the manual published by the company. The procedures for blocking with 5% skim milk solution and the subsequent reactions with antibodies were essentially the same as described earlier (*10*). In some experiments, an ECL kit was employed as indicated.

Treatment with Estradiol-17β—Xenopus females were injected with 200 μl per body of 5 mg/ml estradiol-17β in propyleneglycol, and the liver and ovary were surgically removed on day 0 (control), 8 or 16 after injection. The tissues were homogenized with 5 times the volume of the buffer indicated above (based on individual wet weights) using a Polytron at setting 5 for ovary or setting 6 for liver two times for 30 s each with an interval of the same period. The extract was ultracentrifuged at 165,000 ×*g* for 90 min. The supernatant containing 25 μg of protein and its heat-stable supernatant were used for Western blot analyses for *pNiXa* and pp25, respectively.

Other Procedures and Determinations—Protein was determined by the method of Bradford (*11*) with bovine serum albumin as the standard. The concentration of pp25 was determined as described previously (*1*). Radioactivity was determined with a Beckman LS-5801 liquid scintillation counter with Cerenkov radiation. The amino acid sequence was determined by automated Edman degradation using an Applied Biosystems gas-phase protein sequencer, model 470A, equipped with an on-line reverse-phase chromatography system for the identification of PTH–amino acids.

RESULTS AND DISCUSSION

Overlay Assay of pp25 Binding Protein—In an attempt to examine the binding protein of pp25, overlay analysis was performed on cytosolic, Triton X-100–extracted and Triton X-100–unextracted particulate fractions of *Xenopus* oocytes using ^{32}P-labeled pp25 as a probe. The results in Fig. 1A show one protein that bound radioactive pp 25 with a molecular mass of 43,000 in the cytosolic fraction and several proteins with molecular masses ranging from 43,000 to 17,000 in the Triton X-100–unextracted particulate fraction. In contrast, essentially no binding proteins were detected in the Triton X-100–extracted particulate fraction. In controls in which non-radioactive pp25 was employed, no binding proteins could be detected (data not shown). These results indicate that the radioactive protein bands reflect the binding of ^{32}P-labeled pp25 and not the binding of [γ-^{32}P]ATP. In additional control experiments, an excess amount (100-fold) of non-labeled pp25 was mixed with the radioactive pp25 in the overlay solution. In this case, the intensities of most of the radioactive bands were diminished (Fig. 1B). This result indicates that the binding proteins detected in Fig. 1A do not represent non-specific

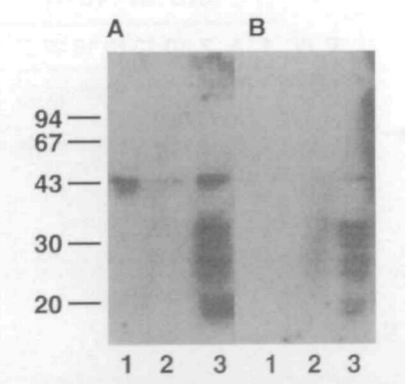

Fig. 1. **Detection of pp25-binding proteins by overlay assay.** Preparation of subcellular fractions and overlay assay were performed according to the methods described under "EXPERIMENTAL PROCEDURES." The numbers on the left side show the positions of molecular mass markers in kDa; 94, phosphorylase *b*; 67, BSA; 43, ovalubumin; 30, carbonic anhydrase; 20, soybean trypsin inhibitor. Lanes 1, 2, and 3 show samples of cytosolic fraction (12 μg of protein), Triton X-100–extracted particulate fraction (11 μg of protein) and Triton X-100–unextracted particulate fraction (11 μg of protein), respectively. A; overlay assay with ^{32}P-labeled pp25, B; overlay assay with ^{32}P-labeled pp25 in the presence of a large excess of non-labeled pp25.

binding of ^{32}P-labeled pp25. The previous report from our laboratory showed the existence of multiple endogenous phosphate in pp25 (about 40–50 mol per mol of this protein) (*2*). An additional 1 or 2 mol of radioactive phosphate incorporated by CK II does not seem to affect seriously the binding ability of pp25. Judging from the diffused peptide mapping pattern, the radioactive phosphate seemed to distribute to multiple sites, rather than to specific site(s) (data not shown). The possibility of proteolytic degradation of ^{32}P-labeled pp25 during the assay was excluded by examining the used overlay solution by SDS-PAGE and autoradiography (data not shown). Although these experiments were performed under *in vitro* assay conditions, the results suggest the possibility of the existence of some targeting proteins for pp25 in *Xenopus* oocytes. A previous report using neutrophils indicated the existence of cytoskeletal proteins in the particulate fraction after extraction with buffer containing 1% Triton X-100 (*12*). There was the possibility of actin as a binding protein with a molecular mass of 43,000 (*13*). However, actin from rabbit skeletal muscle was shown not to be a binding protein of pp25 by the same overlay assay (data not shown).

Purification of p43—In order to identify these binding proteins, at first we selected p43 in the cytosolic fraction because this protein was the only one target detected in this subcellular fraction. The cytosolic fraction (50 ml, 140 mg of protein) stored at −20°C was thawed and centrifuged at 10,000 ×*g* for 10 min to remove insoluble materials. When the supernatant was applied to a DEAE-Sephacel column (2.1 × 11.5 cm) equilibrated with buffer containing 20 mM Tris-HCl, pH 7.5, 10 mM 2-mercaptoethanol, 0.5 mM EGTA, 1 mM PMSF, 47 μM leupeptin, 1 μM pepstatin, 100 μM antipain, 2 μg/ml aprotinin, 60 mM β-glycerophosphate, and 0.1 mM NaF, p43 did not bind to the column and appeared in the pass-through fraction. This solution (87 ml, 80 mg of protein) was applied to a phosphocellulose column (1.5 × 2.5 cm) equilibrated with 20 mM Tris-HCl, pH 7.5, 10 mM 2-mercaptoethanol, 0.5 mM EGTA, 1 mM PMSF, and 47 μM leupeptin. After washing the column with the same buffer containing 250 mM NaCl until the absorbance at 280 nm was reduced to nearly zero, the proteins were eluted with buffer containing 500 mM NaCl, a linear gradient from 500 mM to 1 M NaCl, and 1 M NaCl, as depicted in Fig. 2. Fractions of 1 ml each were collected at a flow rate of 21 ml/h. The results shown in Fig. 3 indicate that p43 was recovered from the column with buffer containing a NaCl level greater than 500 mM in broad fractions. At first, we supposed that the α subunit of

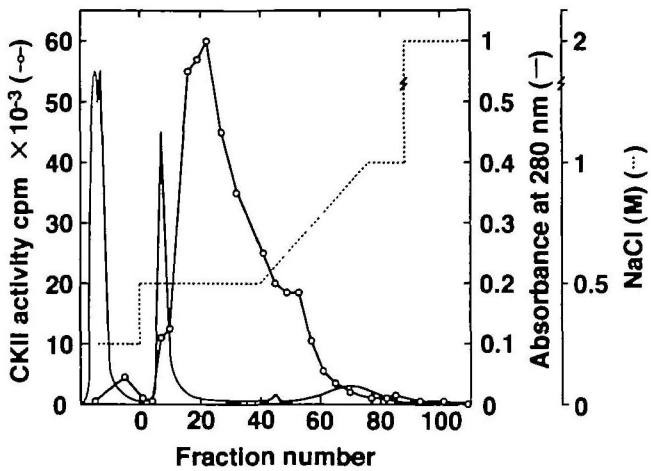

Fig. 2. **Purification of cytosolic p43 by phosphocellulose column chromatography.** Details of the experimental conditions are described in the text. CK II activity was measured with an aliquot of each fraction (30 μl) as indicated under "EXPERIMENTAL PROCEDURES." Solid line, absorbance at 280 nm; dotted line, concentration of NaCl in the buffer; solid line with circles, CK II activity.

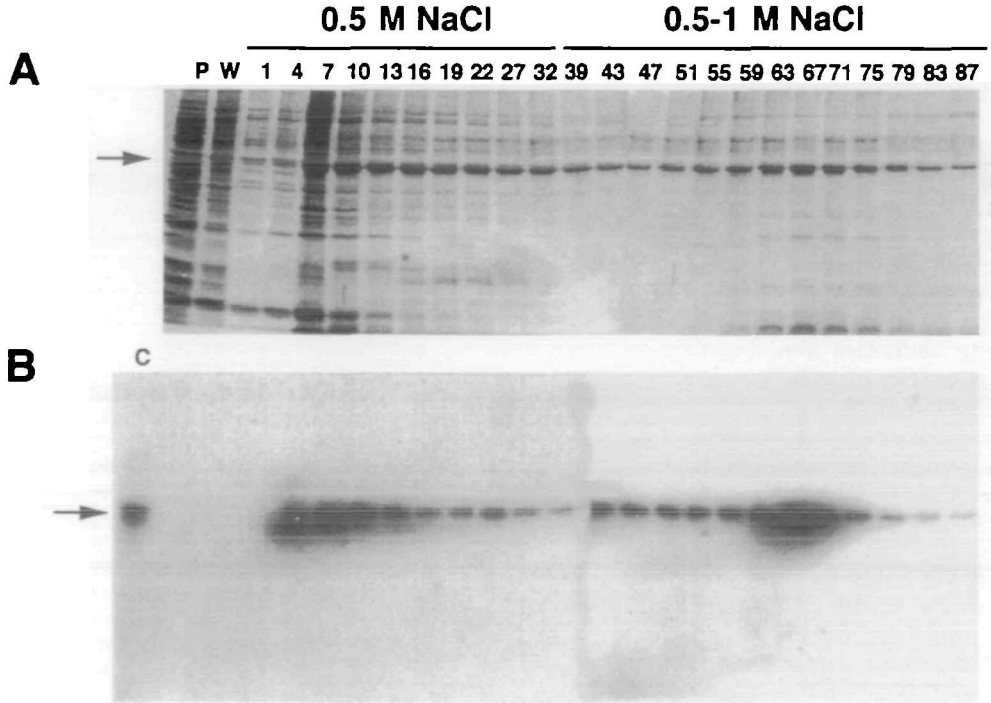

Fig. 3. **Detection of p43 in the fractions eluted from the phosphocellulose column.** SDS-PAGE and overlay assay were performed as described under "EXPERIMENTAL PROCEDURES" using an aliquot (20 μl) of each fraction. P, W, and C show samples of the pass-through fraction, a fraction eluted with buffer containing 250 mM NaCl, and the cytosolic fraction (20 μg of protein), respectively. The numbers indicate fraction numbers from column chromatography. The position of p43 is indicated by the arrows on the left. (A) Coomassie Brilliant Blue staining of the SDS-PAGE gel. (B) autoradiogram of the membrane used for overlay assay. Sometimes, a radioactive band of molecular mass of 42,000 was also detected on the autoradiogram. It is not clear at this time whether this is due to a proteolytic attack of p43 or not.

CK II was a candidate for the pp 25 binding protein judging from its molecular mass (*14*). Although CK II was eluted from the column by buffer containing 0.5 M NaCl, the CK II peak did not coincide with that of the binding protein of ^{32}P-labeled pp25. The α subunit of CK II does not seem to be a target of pp25. The solutions in fractions 10 to 38 (25 ml) and 39 to 99 (60 ml) were separately pooled and concentrated using an Amicon ultrafiltration cell equipped with a YM10 membrane to 1.2 and 1.5 ml, respectively. These concentrated fractions were combined and the NaCl concentration was adjusted to 1 M. This p43 solution was subjected to gel filtration on a Sephacryl S-200 column (2.6 × 96.5 cm) equilibrated with buffer containing 20 mM Tris-HCl, pH 7.5, 10 mM 2-mercaptoethanol, 1 M NaCl, 60 mM β-glycerophosphate, and 0.1 mM NaF. Fractions of 3 ml each were collected at a flow rate of 20 ml/h. The results shown in Fig. 4 indicate that p43 eluted from the column in fractions 78 to 94. The solution obtained (50 ml, 1.2 mg protein) was dialyzed against 1 liter of buffer containing 20 mM Tris-HCl, pH 7.5, and 10 mM 2-mercaptoethanol. This dialysis was repeated 3 times and the solution was applied to a pp25-affinity column (1.3 × 2.8 cm) equilibrated with dialysis buffer. After washing the column with buffer containing 50 mM NaCl until the absorbance of the eluent was nearly zero, the proteins were eluted in a step-wise manner with the same buffer containing 100 mM or 200 mM NaCl. Fractions of 1 ml each were collected at a flow rate of 8.6 ml/h. The results depicted in Fig. 5 indicate that p43 was mainly recovered from the column by buffer containing more than 100 mM NaCl. The concentrations of acetonitrile and trifluoroacetic acid in Fractions 77–79 were adjusted 10 and 0.05%, respectively, and the sample was finally purified on a reverse-phase C18 HPLC column (0.39 × 15 cm, 5μC18-300 Å, Waters) as shown in Fig. 6. The amino acid sequence of a sharp peak eluted by about 40% acetonitrile was analyzed directly, because the protein seemed to bind tightly to the Eppendorf tube and became hard to solubilize in aqueous solution after concentration by freeze-drying. When the solution in Fraction 49 eluted from the affinity column by buffer containing 100 mM NaCl was treated as indicated above and analyzed by HPLC, one major peak was recovered in about 40% acetonitrile, the same as the result shown in Fig. 6 (data not shown). This

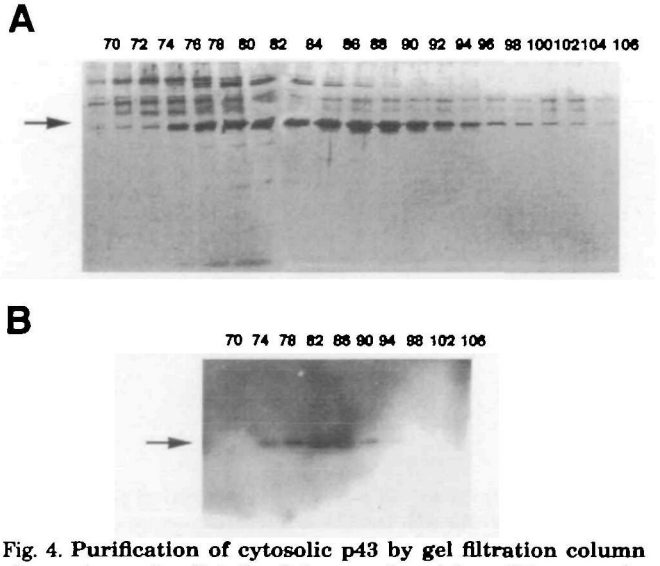

Fig. 4. **Purification of cytosolic p43 by gel filtration column chromatography.** Details of the experimental conditions are described in the text. Other conditions were same as described in the legend to Fig. 3, except that the protein was stained by a silver staining kit in Experiment A and 20 μl aliquots of each fraction were analyzed.

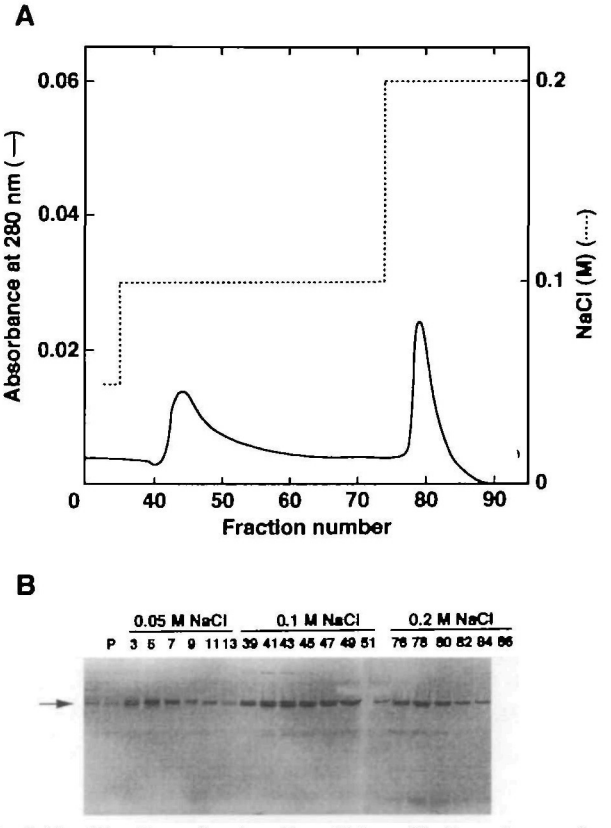

Fig. 5. **Purification of cytosolic p43 by affinity column chromatography of pp25.** Details of the experimental conditions are described in the text. A, protein elution profile. Other indications are same as described in the legend to Fig. 2. B, protein staining pattern by a silver staining kit. P and numbers indicate the pass-through fraction and fraction numbers, respectively.

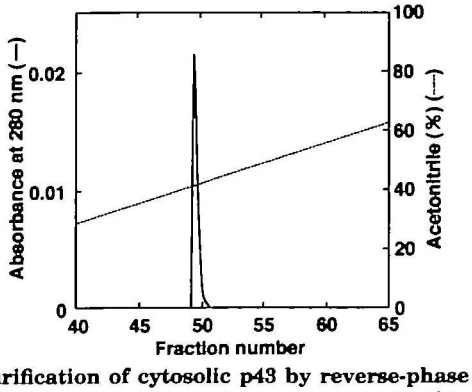

Fig. 6. **Purification of cytosolic p43 by reverse-phase HPLC.** Details of the experimental conditions are described in the text. p43 was eluted with a linear gradient of acetonitrile from 10 to 80% in the presence of 0.05% trifluoroacetic acid. Solid and dotted lines show absorbance at 280 nm and concentration of acetonitrile, respectively.

result suggests that the p43 eluted from the affinity column is nearly homogeneous.

Amino Acid Sequence Analysis of p43—The amino acid sequence of the amino-terminal region of p43 was determined as described in Fig. 7. Data base analysis of this sequence identified p43 as Ep45 whose mRNA is known to be actively synthesized in frog liver after stimulation with estrogen (*5*, *15*). Later, the results of cDNA cloning of this mRNA indicated that this protein seems to belong to a serpin family based on amino acid sequence homology (*5*). On the other hand, this protein has also been identified as an Ni^{2+}-binding protein in *Xenopus* oocytes (*6*). In fact, it has been reported that the original protein synthesized in liver is secreted into the blood and then incorporated into oocytes (*6*, *15*). Consistent with this evidence, the first 16 amino acids of the original sequence are proposed to be a signal peptide (*5*, *6*). The amino-terminal sequence of p43 indicates that the first amino acid, Asp, corresponds to the 24th amino acid of the original sequence (*5*). This result suggests that the 7 amino acids next to the signal peptide are further removed in p43. Beck *et al.* reported that the amino-terminal amino acid is blocked in *pNiXa* (*6*). It is not clear at this time whether this shortening observed in p43 is due to a purification artifact or not. The summary of the amino acid sequence of the amino-terminal region of Ep45 and p43 is presented in Fig. 8.

Immunoblot Analysis of p43—Based on the fact that p43 is same as Ep45 (*5*) or *pNiXa* (*6*), immunoblot analysis was performed using antibody against *pNiXa* (*7*). As shown in Fig. 9, the proteins in the two subcellular fractions used in Fig. 1 were re-examined using this antibody. In addition to the p43 in the cytosolic fraction, p43 in the particulate fraction also cross-reacted with this antibody. Although this result suggests that the particulate p43 is also *pNiXa*, further analysis has not been done. When an aliquot of each fraction recovered from the pp25-affinity column was examined by immunoblot analysis using the *pNiXa* antibody, all of the p43 eluted from the column by buffer containing 50, 100, and 200 mM NaCl reacted with the antibody (data not shown). The reason the p43 that reacted with the antibody was separated into three fractions on this affinity column is not clear at this time. This result and those of the HPLC analyses suggest that most of the p43 detected by affinity column chromatography (Fig. 5) is *pNiXa*. The results shown here suggest a possible interaction between pp25 and p43 (overlay assay in Fig. 1, and affinity column chromatography of pp25 in Fig. 5). Although the antibody against pp25 obtained in our laboratory could be used for Western blotting, it was not suitable for the immunoprecipitation of pp25 (data not shown). In order to obtain further evidence supporting a possible interaction between these proteins, we tried to add biotinylated pp25 to the cytosolic fraction of *Xenopus* oocytes and recover it by immobilized-avidin. The results shown in Fig. 10 indicate that p43 was recovered in the precipitate with biotinylated pp25. If the modified pp25 was omitted, only a trace amount of p43 was

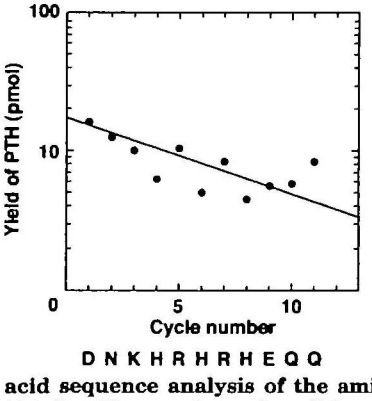

Fig. 7. **Amino acid sequence analysis of the amino-terminal region of p43.** Details of the experimental conditions are described in the text and under "EXPERIMENTAL PROCEDURES." The identified amino acids are indicated at the bottom.

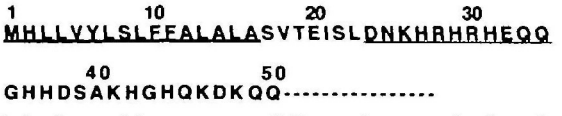

Fig. 8. **Amino acid sequence of the amino-terminal region of Ep45.** The signal peptide of Ep45 (*5*, *6*) and the amino acid sequence of p43 determined in this study are indicated by double and single solid lines, respectively.

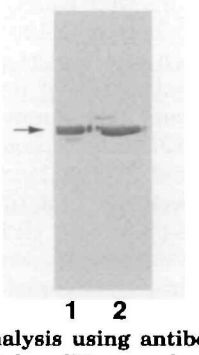

Fig. 9. **Immunoblot analysis using antibody against *pNiXa*.** Details of the experimental conditions are described in the legend to Fig. 1, except that the membrane was incubated with antibody against *pNiXa* and the protein bands were visualized as described in "EXPERIMENTAL PROCEDURES." The arrow shows the position of p43 reacted with the antibody against *pNiXa*. Lane 1, cytosolic fraction; lane 2, Triton X-100–unextracted fraction.

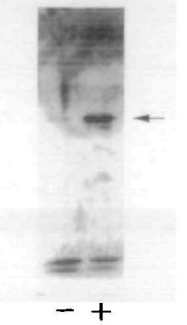

Fig. 10. **Precipitation of p43 with biotinylated pp25 and immobilized avidin gel.** Details of the experimental conditions are described under "EXPERIMENTAL PROCEDURES." After SDS-PAGE, the proteins were transferred to a PVDF membrane, and p43 was visualized by an immunoblot procedure using the antibody against *pNiXa* and an ECL kit. The arrow shows the position of p43. The cytosolic fraction of oocytes was incubated without (−) or with (+) biotinylated pp25.

recovered by this avidin-gel. This result also supports the idea that p43 is a candidate targeting protein.

Mechanism of pp25 Binding to pNiXa—As to the mechanism of binding of pp25 and *pNiXa*, the interaction between the cluster of histidine residues in the amino-terminal region of *pNiXa* and the anionic surface due to the multiple phosphorylated amino acids in pp25 seems to be important (*2*, *5*, *16*). At first, the role of these phosphates was examined. The most of the endogenous phosphate was removed by treatment with acid phosphatase, which converted pp25 to the protein with molecular mass of 20,000, as reported earlier (*2*). This latter protein was rephosphorylated by CK II to a level of about 4 mol of phosphate per mol of protein and used as the probe for the poorly phosphorylated protein. By this treatment, the phosphate level was decreased to less than 10% of that of usual pp25 (*2*). As shown in Fig. 11, it became hard for the dephosphorylated form of pp25 to bind *pNiXa*. This result suggests that the multiple endogenous phosphate of pp25 is important for the interaction with *pNiXa*. In the next experiment, the role of histidine residues in *pNiXa* was examined. By limited proteolysis with trypsin, the amino-terminal region of *pNiXa*, containing the cluster of histidine residues, was removed (*7*) and the native and the digested samples were subjected to overlay assay. The removal of the histidine residues was confirmed by Western blotting using *pNiXa* antibody (*6*, *7*). The results shown in Fig. 12 indicate that the *pNiXa* fragment lacking the histidine residues could not bind pp25. These results suggest that the interaction between the histidine cluster in *pNiXa* and the multiple phosphorylated amino acids in pp25 is important for the binding of these proteins. Consistent with these results, a previous report has indicated that the histidine-glycine-rich region in the light chain of high molecular weight kininogen serves as a primary structural feature for zinc-dependent binding to anionic surfaces (*17*).

Effect of pp25 on Serpin Activity of pNiXa—Ep45 or *pNiXa* has been shown to belong to a family of serpins based on the amino acid sequence and the inhibitory effect on various serine protease activities (*5*–*7*). In the last set of experiments, the effect of pp25 on the serpin activity of *pNiXa* was examined. The results shown in Fig. 13 indicate that pp25 added at a concentration similar to that of *pNiXa* did not seriously influence the serpin activity of *pNiXa* toward α-chymotrypsin. Preincubation of pp25 with *pNiXa* overnight at 4°C or the addition of a 10-fold higher concentration of pp25 also did not clearly affect the action of *pNiXa* (data not shown).

In this report, pp25 binding proteins were analyzed in order to obtain some clue as to the function of this protein. Based on the partial amino acid sequence and immunoblot analyses (Figs. 7–9), we identified a cytosolic p43 as Ep45 (*5*) or *pNiXa* (*6*). The slight difference in molecular masses in these studies is due mainly to regarding the mass of ovalubumin as 43,000 (this paper) or 45,000 (*7*). After purification by HPLC, p43 became hard to solubilize with aqueous solutions by freeze-drying. This may be due to the relatively high content (about 40%) and clusters of hydrophobic amino acids detected in Ep45 or *pNiXa* (*5*, *6*). The properties of *pNiXa* were studied extensively by Dr. Sunderman's group (*6*, *7*, *16*). It seems to be important to examine the effect of pp25 on other functions of *pNiXa*. It has been reported that *pNiXa* is one of three Ni-binding pro-

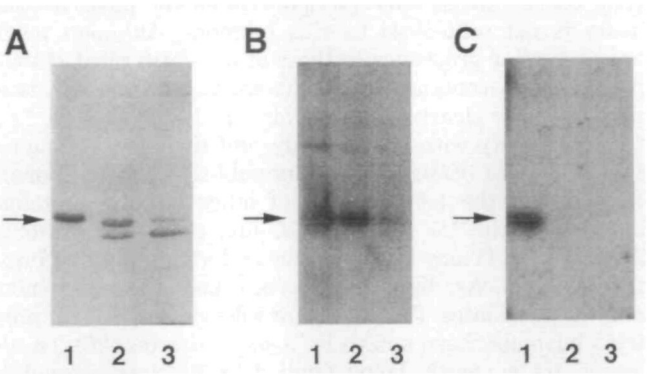

Fig. 12. **Role of the histidine-rich region in the amino-terminal domain of *pNiXa* for binding with pp25.** Overlay assay of *pNiXa* subjected to limited proteolysis with trypsin for 0 min (lane 1), 10 min (lane 2), and 30 min (lane 3) was performed using ^{32}P-labeled pp25 according to the usual method described under "EXPERIMENTAL PROCEDURES." The arrow shows the position of p43. (A) protein staining; (B) Western blotting with the antibody against *pNiXa*; (C) overlay assay.

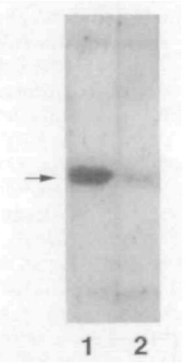

Fig. 11. **Role of multiple endogenous phosphate in pp25 for binding with *pNiXa*.** Overlay assay of *pNiXa* was performed using pp25 (lane 1) and dephosphorylated pp25 (lane 2), both of which were ^{32}P-labeled with CK II under the conditions described under "EXPERIMENTAL PROCEDURES." The arrow shows the position of p43.

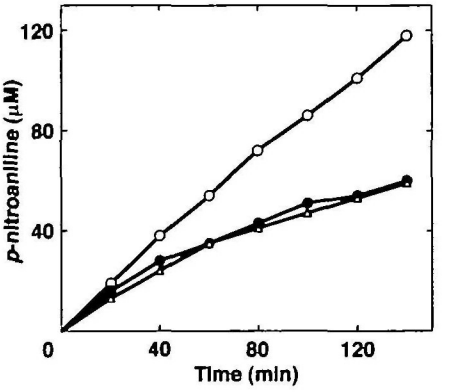

Fig. 13. **Effect of pp25 on the serpin activity of *pNiXa*.** The time course of α-chymotryptic activity was measured under the conditions described under "EXPERIMENTAL PROCEDURES" except that 64 nM pp25 and 160 nM *pNiXa* were added as indicated below. (○) in the presence of pp25 and *pNiXa* buffer; (●) in the presence of *pNiXa* and pp25 buffer; (△) in the presence of *pNiXa* and pp25.

teins detected in *Xenopus* oocytes and also found in embryos (*6*, *16*). Recently, pp25 was also detected in embryos in our laboratory and shown to disappear around Nieuwkoop/Faber stage 40 (Funami, J., unpublished observation), as in the case of *pNiXa* (*6*). A recent report indicated that Ep45 blocks the ability of follistatin to inhibit bone morphogenic protein activity and to induce a secondary body axis (*18*). However, the physiological role of the interaction between pp25 and *pNiXa* (or Ep45) is not yet solved. The quantitative relationship between these proteins in oocytes is not clear at this time, because the intracellular concentration of each protein has not been determined exactly. It has been reported that the level of the mRNA for Ep45 increases signifcantly in frog liver around 2 weeks after the injection of estradiol-17β (*15*). We also examined the quantitative changes in *pNiXa* and pp25 by immunoblotting after stimulation with this hormone. The amount of *pNiXa* increased in liver, but those of *pNiXa* and pp25 in ovary did not change significantly (data not shown). The pp25 was detected consistently at the position corresponding to its fully phosphorylated form, as before. This result suggests that the synthesis and phosphorylation of pp25 in the ovary is not influenced by this hormone. Although pp25 shows similar properties to those of phosvitin (heat stability and high content of phospho-amino acids), these two proteins differ clearly in their amino acid sequences (*1*, *19*), their reactivity with the antibody, and their protein staining properties (Kobayashi, N., unpublished observations). In addition, the identification of other binding proteins detected in the 1% Triton X-100–unextracted particulate fraction (Fig. 1) may be helpful for understanding the function of pp25. As discussed above, cytoskeletal protein(s) may be candidates. The important roles of cytoskeletal proteins have also been shown in *Xenopus* oocytes (*20*). These issues are currently being studied in our laboratory. We have not yet succeeded in cloning the cDNA of pp25, but the analysis of the binding protein by a yeast two hybrid system is also one of our future projects, as well as the verification of the co-localization of pp25 and *pNiXa* using highly specific antibodies.

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