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Phosphorylation of the 24p3 Protein Secreted from Mouse Uterus *in Vitro* and *in Vivo*

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The 24p3 protein is a 25 KDa glycoprotein, having been purified from mouse uterine fluid. Thr^{54}, Ser^{88}, and Thr^{128}/Ser^{129} on the protein molecule were predicted to be the phosphorylation site of casein kinase II, protein kinase C, and cAMP-dependent protein kinase, respectively. Incorporation of phosphate to this protein from [γ-^{32}P]-ATP was tested in the solution suitable for the three kinases. Neither casein kinase II nor cAMP-dependent protein kinase reacted to the 24p3 protein; however, protein kinase C demonstrated phosphorylation to this protein. This phosphorylation may be competing with a polypeptide segment: Arg^{79}-Tyr-Trp-Ilu-Arg-Thr-Phe-Val-Pro-Ser^{88}-Ser-Arg-Ala-Gly-Gln-Phe-Thr-Leu-Gly^{97} in the 24p3 protein molecule. To support this theory, Ser^{88} is a phosphorylation site of protein kinase C on 24p3 protein. The enzyme kinetic parameter, based on the Michaelis-Menten equation, determined Km to be 2.96 μM in the phosphorylation of 24p3 protein by the kinase. Both of the phosphorylated and dephosphorylated form of 24p3 protein can enhance the cAMP-dependent protein kinase activity *in vitro.* In addition, this experiment will show for the first time that serine-phosphorylated 24p3 protein exists in mouse uterine tissue.

KEY WORDS: Phosphorylation; protein kinase; serinephosphate; uterine protein.

1. INTRODUCTION

A purified protein, derived from 24p3 cDNA, was originally by Hraba-Renevey *et al.* (1989) cloned from mouse kidney culture cells infected with polyoma virus-40, from mouse uterine luminal fluid (Chu *et al.,* 1996), and identified in the epididymis (Chu *et al.,* 2000). We identify this protein as 24p3, a 25 KDa glycoprotein, with a blocked N-terminus of pyroglutamate (Chu *et al.,* 1997). This protein is also present in lipopolysaccharide-stimulated PU5.1.8 macrophage (Meheus *et al.,* 1993) and bFGF-stimulated 3T3 cells (Davis *et al.,* 1991). The results of Liu and Nilson-Hamilton (1995) reveal it as an acute phase protein of liver. Based on a computer-assisted homologous search, it is classified as a member of the lipocalin superfamily (Flower *et al.,* 1991). The protein also shows a high degree of similarity to human neutrophil gelatinase-associated lipocalin (NGAL) (70% identity) (Chu *et al.,* 1997; Kjeldsen *et al.,* 1993). Our previous results support a hydrophobic pocket of this protein molecule, showing it to be suitable for fatty acid and retinoid binding (Chu *et al.,* 1998). We also found that the epididymal 24p3 protein interacted predominantly with the acrosome of spermatozoa (Chu *et al.,* 2000). The biological function of this uterine protein remains unclear. This being evident, we are seeking further understanding of the function of the 24p3 protein.

Phosphorylation and dephosphorylation of protein is well-recognized as an important to regulate biological function: It may reflect a relationship between protein structure and biological activity. The phosphorylation or dephosphorylation of amino acid residue triggers struc-

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[4] Abbreviations: CKII, casein kinase II; NC, nitrocellulose; NGAL, neutrophil gelatinase-associated lipocalin; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; PMSF, phenylmethyl sulfonyl; TFA, trifluoroacetic acid.

tural change in the molecules, altering their biological function (Kurosawa, 1994). The analysis of the primary structure of 24p3 protein suggests the presence of potential Ser/Thr sites for the phosphorylation by casein kinase II (CKII),[4] protein kinase C (PKC), or cAMP-dependent protein kinase (PKA) (Fig. 1), thus further emphasizing the importance of verifying the phosphorylation in 24p3 protein. Accordingly, we investigated the phosphorylation of 24p3 protein by these three types of kinases.

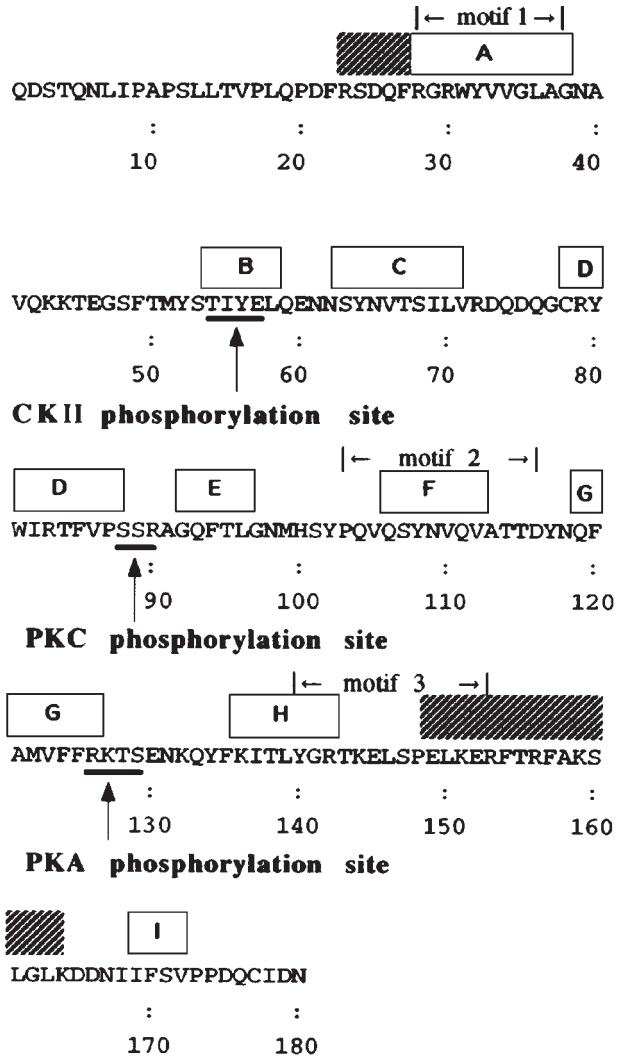

Fig. 1. The potential phosphorylation sites of protein kinases on 24p3 protein and its predicted secondary structure. Based on our previous report (Chu *et al.*, 1998), the predicted secondary structures, which are conserved in the lipocalin protein superfamily (Monaco and Zanotti, 1992), are shown at the top of the sequence (β-strands A–I and helices in the hatch blocks). The three short motifs, which are highly conserved between members of the family (Flower *et al.*, 1991) are denoted. The consensus sequences for the phosphorylation site of different protein kinases are underlined and denoted.

Our results support a PKC phosphorylation site at Ser^{88} but exclude both phosphorylation sites for CKII and PKA on the protein molecule. The Km value is comparable to that reported for the most effective protein substrates for protein kinase C (Abe *et al.*, 1991). Use of the immunoprecipitation method to identify the serine-phosphorylated 24p3 protein in uterine tissue indicated the phosphorylation of 24p3 protein might be important and further established that the 24p3 protein enhanced the PKA activity *in vitro.*

2. MATERIALS AND METHODS

2.1. Materials

Outbred ICR mice were purchased from Charles River Laboratory (Wilmington, MA) and were bred in the animal center at the College of Medicine, National Taiwan University. All enzymes were purchased from Boehringer Mannheim G.m.b.H (Germany). Antiphosphoserine antibody and HRP-conjugated anti-rabbit IgG were obtained from Zymed Laboratories, Inc. (Cat no. 61-8100, CA. USA) and Promega (cat no. W4011, WI, USA), respectively. The 24p3 protein-induced antibody was prepared and partially purified with a protein A column, as per a previous method (Chu *et al.*, 2000). The [γ-^{32}P]-ATP was from Amersham (Searle, Arlington Height, IL). All other chemicals were reagent grade.

2.2. Prediction of Phosphorylation Sites

The method of matching consensus sequence patterns based on stepwise discrimination analysis was applied to seek the potential phosphorylation sites on the 24p3 protein molecule from a primary structure of the 24p3 protein. The analysis was executed by the computer program package PROSITE 9.1, which is (technically) interrelated to SWISS-PROT protein sequence data bank (Bairoch and Apweiler, 1997, 1998).

2.3. Solid-Phase Peptide Synthesis

Peptide was synthesized, via 4-(2,4-dimethoxyphenyl-Fmoc-amino-methyl) phenoxyl resin (0.281g, 0.89 mEq/g) with Fmoc-amino acid derivatives using an automatic peptide synthesizer (Applied Biosystem Model 433A, USA). After completion of synthesis, the peptide on resins were incubated with a cleavage mixture containing 0.75 g crystalline phenol, 0.25 mL 1,2-ethandithol, 0.5 mL thioanisole, 0.5 mL D.I. water, 10 mL trifluoro-

acetic acid, for 90 min at room temperature, and the solvent was completely evaporated. The dry resin was then washed five times with 20 mL of cold ether. Synthetic peptide was then extracted by washing five times with 20 mL of 5% acetic acid. All extracts were lyophilized to yield a crude peptide (0.506g, 93.5% yield).

2.4. *In Vitro* Phosphorylation of 24p3 Protein

The [γ-^{32}p]-ATP was diluted with unlabeled ATP, yielding a final specific activity of 0.5 Ci/mmol. In a specified solution reported previously (Gonzalez *et al.*, 1993; Hathaway and Traugh 1979; Glover *et al.*, 1983), 24p3 protein (5.0 μg/mL) was phosphorylated by CKII (20 mU/mL), PKA (20 μU/mL), or PKC (2 μU/mL) in the presence of 0.2 nM radial labeled ATP at 30°C for 5 min. The phosphorylation by PKC was processed in 20 mM HEPES containing 15.0 μM phosphatidylserine, 1.3 mM $CaCl_2$, 10 mM $MgCl_2$, and 1.0 mM DTT at pH 7.4. The PKA phosphorylation was performed in 50 mM MES containing 10 mM $MgCl_2$, 0.5 mM EDTA, and 1.0 mM DTT at pH 6.9. The CKII phosphorylation was carried out in 20 mM MES containing 130 mM KCl, 10 mM $MgCl_2$, and 4.8 mM DTT at pH 6.9. The reaction mixtures were subjected to SDS/PAGE on a 15% polyacrylamide slab gel (0.075 × 5.0 × 6.0 cm). The gel was dried by a gel-dryer, then autoradiographed on x-ray film.

2.5. The Phosphorylation Activity of PKC and PKA

The PKC activity was assayed according to a phosphocellulose technique (Casnellie, 1991). In the specified solution, 24p3 protein (0–14 μM), or synthetic polypeptide (0–2 μM) comprising Arg^{79}-Tyr-Trp-Ilu-Arg-Thr-Phe-Val-Pro-Ser^{88}-Ser-Arg-Ala-Gly-Gln-Phe-Thr-Leu-Gly^{97}, of the protein molecule was incubated in the presence of PKC (2 μU/mL) and 0.2 nM [γ-^{32}P]-ATP at 30°C. The 10 μL of aliquot was spotted onto a P81 phosphocellulose paper for 5 min and then baked for 40 min under a lamp. The paper was washed five times with 2.0 mL of 150 mM phosphoric acid, rinsed with alcohol for 2 min, and baked dry. The radioactivity on the papers was counted by a β-counter. The velocity of ^{32}P-incorporation to the protein substrate was fitted to a double reciprocal plot constructed from the Michaelis-Menten equation.

The established method of Goueli *et al.* (1995), the cAMP-dependent protein kinase activity, was carried out in a 25 μL reaction mixture containing 40 mM Tris-HCl, pH 7.4, 20 mM $MgCl_2$, 100 μM [γ-^{32}p]ATP, 100 μg/mL BSA, and 100 μM biotinylated peptide substrate (Leu-Arg-Arg-Ala-Ser-leu-Gly) (V7480, Promega, WI, USA). The reaction mixture was preincubated at 30°C for 5 min, followed by 37°C for 5 min. At each treatment, a 10-μL aliquot was spotted on a streptavidin-conjugated membrane and washed with 2 M NaCl. This washing process was repeated four times, followed by an additional four times washing with 1% H_3PO_4 containing 2M NaCl, and twice with distilled water to remove the free isotope impurities. The membranes were then dried and counted using a liquid scintillation counter.

2.6. Phosphorylation and Dephosphorylation of 24p3 Protein

The purified 24p3 protein of uterine luminal fluid was prepared according to our previous method (Chu *et al.*, 1996). Fifty μg of 24p3 protein was mixed with 300 μU of bovine placenta alkaline phosphatase in 200 μL 0.1 M glycine, pH 10.4, containing 0.1 M NaCl, 50 mM $MgCl_2$, 1.0 mM DTT, 2.0 mM EDTA, and 100 μM phenylmethyl sulfonyl fluoride (PMSF). The mixture was incubated at 30°C for 4 h to remove the phosphate from protein. After the dephosphorylation, 60 μL 0.2 N HCl was added to neutralize and the mixture was fractionated by reversed-phase HPLC, with a 35% to 45% linear gradient of acetonitrile in 0.1% trifluoroacetic acid (TFA), and a flow rate of 0.7 mL/min on a C18, 7 micron, 250 × 4.6 mm column (Macherey-Nagel, GmbH & Co., KG). The dephosphorylated 24p3 protein was collected manually and lyophilized.

To phosphorylate the protein, 30 μg 24p3 protein was mixed with 50 ng PKC in 100 μL of 20 mM Tris-HCl, pH 7.5, containing 10 mM $MgCl_2$, 0.2 mM $CaCl_2$, 3 μg/μL phosphatidylserine, 1 μg/μL 1,2-dioleoyl-sn-glycerol, 100 μM Na_3VO_4, and 100 μM PMSF. The reaction mixture was incubated at 37°C for 1 h to ensure the phosphorylation reaction. After the reaction, the phosphorylated 24p3 protein was fractionated by reversed-phase HPLC, as with the previous method. Phosphorylated 24p3 protein was then collected manually and lyophilized. The dephosphorylation/phosphorylation 24p3 protein was analyzed by Western-blot analysis with an antiphosphoserine antibody.

2.7. *In Vivo* Phosphorylation Determination

Mature female mice (6 to 8 weeks) at proestrus phase were sacrificed by cervical dislocation and their uteri removed. The uteri were homogenized in a solution

containing 20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 25 mM NaF, 0.1 μM okadic acid, 2 mM Na_3VO_4, and 2.5 mM β-glycerophosphate to extract the proteins, and then centrifuged at 360,000 × *g* for 40 min to remove the debris. The extraction mixture also contained 1X dilution of protease inhibitor cocktail, (PI Cocktail, Cat No. 1697498, Roche Molecular Biochemicals, Germany). The supernatant was then obtained as a tissue homogenate extract. Using the Dynabeads immunomagetic system to isolate the 24p3 protein from tissue extract, a target antigen-antibody complex can be isolated with an indirect technique. With the indirect technique, a sheep anti-rabbit IgG, specific for the IgG-antigen complex, is coupled to the Dynabeads M-280 (Dynal, cat No. 112.03, Dynal A. S, Oslo, Norway). The coated beads are then used to capture the desired target of protein complex. The homogenate extract containing 24p3 protein (3 mg) is incubated with the 24p3 protein-induced antibody (3 μg) for 2 h at 4°C, while gently agitating with a rotator. This procedure allows for the antigen-antibody complex formation to be completed. The antigen-antibody complex can now be isolated by incubation with 50 μL Dynabeads M-280 sheep anti-rabbit IgG for 1 h at 4°C. After the incubation, a magnet is applied on the wall of the test tube for 1 min, isolating the target. A 10 μL electrophoresis sample buffer is added and boiled for 5 min, and then centrifuged at 6000 × *g* for 1 min to remove the beads. The dissolved proteins in the sample buffer were resolved by SDS/PAGE [15% (w/v) acrylamide] on a gel slab. Proteins were transferred from gel to a nitrocellulose (NC) membrane in PBS at 4 °C for 32 h by the diffusion method (Bowen *et al.*, 1980). The transferred proteins were detected with antiphosphoserine antibody (diluted to 1.25 μg/10 mL), or 24p3 protein antibody (diluted to 2 μg/15 mL), followed by HRP-conjugated anti-rabbit IgG diluted to 1:12000 and fluorography. The reactive bands were visualized using an enhanced chemiluminescene (ECL) kit (RPN 2132, Amershan Pharmacia Biotech U.K. Ltd) and exposed on x-ray film.

3. RESULTS

3.1. Phosphorylation of 24p3 Protein by Kinases

Fig. 1 shows the motif search for the consensus sequences of protein kinase phosphorylation sites on the 24p3 protein molecule. Based on our previous report (Chu *et al.*, 1998), both the predicted secondary structure, including the three short motifs, are highly conserved in the lipocalin protein superfamily (Monaco and Zanotti, 1992), and are deciphered in Fig. 1. The Thr^{54}-Ile-Tyr-Glu^{57}, Ser^{88}-Ser-Arg^{90}, and Arg^{126}-Lys-Thr-Ser^{129} matched with the consensus sequences for the phosphorylation site of CKII, PKC, and PKA, respectively. The three peptide segments are not included in the three short, highly conserved lipocalin family motifs. The first one is inside β-strand B, the second in a less rigid conformation and may be in a β-turn or in unordered region between β-strands D and E. Part of the third is overlapped with β-strand G. The ability of these protein kinases to phosphorylate Thr^{54}, Ser^{88}, and Thr^{128}/Ser^{129} was examined. The progressive result of the ^{32}P-incorporation from [γ-^{32}P]-ATP to the proteins in the specified solution for each kinase at 30°C for 5 min is determined by β-counter. Fig. 2A displays the

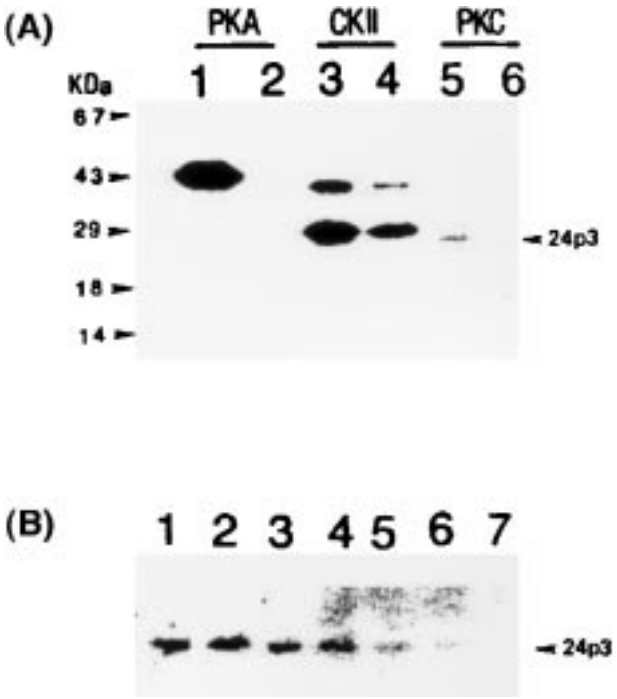

Fig. 2. *In vitro* phosphorylation of 24p3 protein by kinases. (**A**) Each kinase was incubated alone (lanes 2, 4, 6) or in the presence of the 24p3 protein (lanes 1, 3, 5) and the ^{32}P-incorporation to the protein (20 μM) was detected on the autoradiogram after the reaction mixture had been resolved by SDS/PAGE on a 15% polyacrylamide gel slab (see text for details). (**B**) 24p3 Protein (20 μM) was incubated with PKC (2 mU/mL) in the presence of [γ-^{32}P]-ATP (0.5 Ci/mmol) and a synthetic polypeptide comparing Arg^{79}-Tyr-Trp-Ilu-Arg-Thr-Phe-Val-Pro-Ser^{88}-Ser-Arg-Ala-Gly-Gln-Phe-Thr-Leu-Gly^{97} in the protein at 30°C for 5 min. The reaction mixture was resolved by SDS/PAGE and the extent of ^{32}P-incorporation to the 24p3 protein was compared following autoradiography. The molar ratio of the synthetic polypeptide to the 24p3 protein in the incubation was: 0 (lane 1), 1.0 (lane 2), 2.0 (lane 3), 5.0 (lane 4), 10.0 (lane 5), 20.0 (lane 6), 40.0 (lane 7).

autoradiogram of ^{32}P-labeled proteins after the reaction mixtures were resolved by SDS/PAGE; these protein kinases could be distinguished by their migration on SDS/PAGE. PKA appeared as a 41 kDa band and PKC as a 82 kDa band. CKII was resolved into one 42 kDa α-subunit and one 26 kDa β-subunit. Neither PKA nor PKC but both subunits of CKII became radiolabeled when each kinase was isolated and incubated (cf. lanes 2, 4, and 6, of Fig. 2A), indicating that the autophosphorylation occurred only at both subunits of CKII among the three protein kinases. PKA showed no activity to phosphorylate 24p3 protein but the kinase became phosphorylated by the presence of 24p3 protein during incubation (cf. lanes 1 and 2, of Fig. 2A). The autophosphorylation of both subunits of CKII was enhanced by 24p3 protein, although the enzyme did not phosphorylate 24p3 protein (cf. lanes 3 and 4 of Fig. 2A). One radiolabeled protein band corresponding to 24p3 protein was detected in the incubation of PKC with 24p3 protein (cf. lanes 5 and 6 of Fig. 2A), indicating that PKC is able to phosphorylate 24p3 protein. Because Ser^{88} is a predicted phosphorylation site of PKC, the interaction between PKC and a synthetic polypeptide comprising Arg^{79}-Tyr-Trp-Ilu-Arg-Thr-Phe-Val-Pro-Ser^{88}-Ser-Arg-Ala-Gly-Gln-Phe-Thr-Leu-Gly^{97} in the 24p3 protein molecule was further investigated. The ^{32}P-incorporation of [γ-^{32}P]-ATP to 24p3 protein is suppressed by the presence of this polypeptide. The results of one representative experiment (shown in Fig. 2B) indicates a decrease 24p3 protein phosphorylation coincided with the increase in the molar ratio of the polypeptide to 24p3 protein in the reaction. When the molar ratio was more than 20, the 24p3 protein was unable to be phosphorylated.

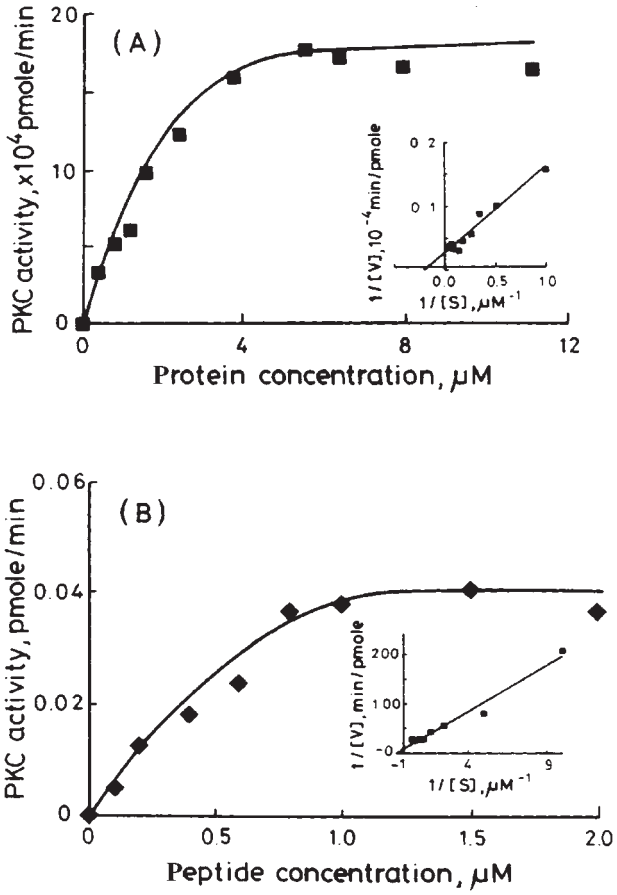

Fig. 3. The kinetics of PKC for the phosphorylation of 24p3 protein. The 24p3 Protein (0–14 μM) or the synthetic polypeptide (0–2 μM) was incubated with PKC (2 μU/mL) in the presence of [γ-^{32}P]-ATP at 30°C. The rate of ^{32}P-incorporation to 24p3 protein (**A**) or the synthetic polypeptide (**B**) was measured. The double reciprocal plot based on the Michaelis-Menten equation is given in the inset.

3.2. Phosphorylation Kinetic of PKC on the 24p3 Protein

The velocity of phosphate incorporation from ATP to the protein substrates by the PKC phosphorylation has been determined (Fig. 3A), and the kinetic parameters were obtained from the double reciprocal plot, based on Michaelis-Menten equation (Fig. 3A, inset). Km was estimated at 2.96 and 0.42 μM for phosphorylation of 24p3, or the synthetic polypeptide, respectively (Fig. 3B). It is surprising to note that the phosphorylation of the synthetic polypeptide occurs more rapidly than that of the 24p3 protein due to the phosphorylation site on the synthetic polypeptide being less rigid, and it becomes more susceptible to the enzyme catalysis.

3.3. The 24p3 Protein Enhances PKA Activity

Fig. 4 shows the dose-relationship activity for PKA in the presence of the 24p3 protein. Comparing the PKA activity in the absence of 24p3 protein solution (Fig. 4 □) with the presence of phosphorylated (Fig. 4 ■), or unphosphorylated 24p3 protein (Fig. 4 ☑) in the incubation medium, sufficiently increased the PKA activity by 1.25- to 5-fold, because the 24p3 protein molar ratio was increased by 10- to 100-fold (Fig. 4A–E). Although the PKA activity in the presence of dephosphorylated 24p3 protein showed few enhancements than in the presence of phosphorylated form, both forms enhanced he PKA activity.

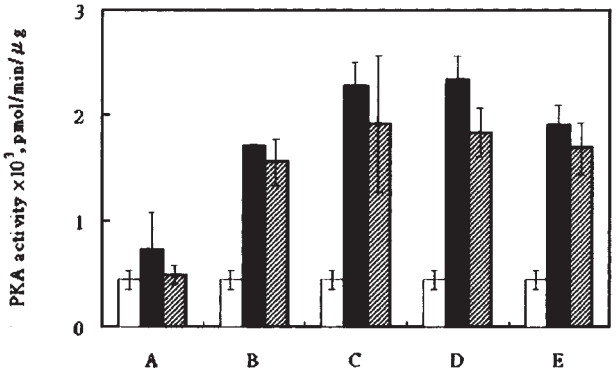

Fig. 4. Effect of 24p3 protein on the PKA activity. The 0.25 pmole PKA activity was determined (cf. Methods) in absence (□) or presence of phosphorylated (■) or unphosphorylated 24p3 protein (☑). Amount of added 24p3 protein: A, 2.5 pmole; B, 5.0 pmole; C. 10.0 pmole; D, 12.5 pmole; E, 25.0 pmole. Each value represents the mean ± SEM of triplicates from a representative experiment.

3.4. Detection of Phosphoserine in 24p3 Protein from Mouse Uteri

The 24p3 protein can be phosphorylated *in vitro* by PKC at the same site as observed *in vivo*, as judged by Dynabeads immunoprecipitation. The serinephosphate on the NC membrane has shown that the 24p3 protein in the immunoprecipitate is phosphorylated *in vivo* (lane 2, Fig. 5B). As shown in Fig. 5 (panel A), the precipitated 24p3 protein was verified by 24p3 protein induced antibody at uterine tissue (lane 2) and purified 24p3 protein as a control (lane 1). Comparing the result of lane 1 with lane 2 in Fig. 5B, the phosphorylated-serine is absent in the purified-24p3 protein; dephosphorylation might occur in uterine luminal fluid or during purified process. The weakly fluorescent band of anti-rabbit IgG, shown in panel C (Fig. 5B), indicates the antiphosphoserine antibody reacted specifically to phosphoserine of the 24p3 protein. Using alkaline phosphatase to dephosphorylate the precipitate taken from uterine tissue with an antibody for the 24p3 protein showed only a dim fluorescent band by the antiphosphoserine antibody, which supports the existence of *in vivo* phosphorylation of the 24p3 protein (data not shown).

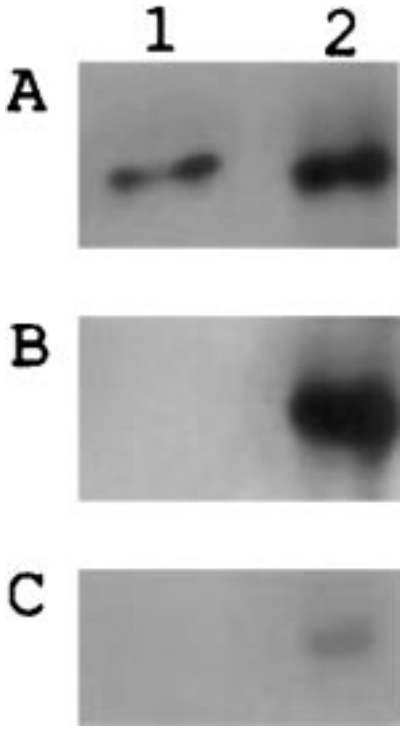

Fig. 5. Immunodetection of phosphorylated 24p3 protein in uterine tissue. Mouse uterine tissues were collected (cf. Methods). The 24p3 protein was immunoprecipitated from uterine tissue and analyzed by Western-blot analysis with antibody against 24p3 protein or phosphoserine. Lane 1 is purified 24p3 protein as a control. Lane 2 is the immunoprecipitated protein from mouse uterine tissue. Panel A, detected by 24p3 protein induced antibody; panel B, detected by antiphosphoserine antibody; panel C, detected by anti-rabbit IgG antibody as a control.

4. DISCUSSION

Our observations have shown that an apparent Km in the PKC phosphorylation of the 24p3 protein (2.96 μM) is comparable to that hitherto reported as the most effective protein substrates for PKC (0.3–9.7 μM) (Abe *et al.*, 1991). The likelihood of phosphorylating 24p3 protein by PKC *in vivo* is conceivable. Our study of *in vitro* and *in vivo* phosphorylation suggests PKC phosphorylation suggests PKC phosphorylation on phosphoserine of 24p3 protein does exist in this uterine protein. The result shown in Fig. 2A, lane 1; suggests that the 24p3 protein induces the conformational change of PKA and facilitates the autophosphorylation of the PKA. The phenomenon of autophosphorylation has been noted in many protein kinases, such as cAMP-dependent protein kinase II (Rubin and Rosen, 1975). The potential role of such autophosphorylation to alter activity of the kinase has been suggested in many reports. The enhancement of enzymatic activity may result from the 24p3 protein association, thus causing the conformational change of PKA. Based on the results (Fig. 2, lane 1; Fig. 4), it is reasonable to speculate that the 24p3 protein may have induced the conformational change of PKA to facilitate the autophosphorylation and then induced the enzymatic activity. The phenomenon of phosphorylation on 24p3 protein may not correlate to PKA activity directly. In the present study, the CKII activity was not affected by 24p3 protein (data not shown),

even if the 24p3 protein increased autophosphorylation of CKII. Elucidation of the interrelationship between 24p3 protein and PKA is more worthy to elucidate. Further studies are necessary to determine if enhancement of PKA activity will occur *in vivo.*

In summary, we show for the first time that 24p3 protein is a serine phosphorylated-glycoprotein in mouse uteri. The phosphorylation and glycosylation, the post-translational modifications of a protein, which have optional, affect the activity of secretion (Kukuruzinska and Leman, 1998; Price *et al.*, 1994). As a secretory protein, whether phosphorylation (in this paper) or glycosylation (Chu *et al.*, 1996) of the 24p3 protein are a necessary requirement for the secretion of the 24p3 protein from the tissue will require future study. Because the phosphorylation/dephosphorylation of a lipid-binding protein may regulate the directional orientation of lipid flux in cells (Buelt *et al.*, 1992) as a lipocalin of 24p3 protein (Chu *et al.*, 1997), the study of phosphorylation of the 24p3 protein is important. Phosphorylation may represent a mechanism, which regulates a 24p3 protein association with the hydrophobic molecule and regulates biological activity of the 24p3 protein. The outcome may determine how the phosphorylation role is identified in the hydrophobic molecule interaction or protein secretion. Based on the result of the autoradiogram study, the fact that the 24p3 protein facilitated the autophosphorylation of PKA and enhanced the PKA activity, indicating an interrelation between the 24p3 protein and kinase *in vivo*, merits further attention. Further study is needed to elucidate the significance of the 24p3 protein within the reproductive system.

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