Heat-shock protein-25/27 phosphorylation by the δ isoform of protein kinase C

Evelyn T. MAIZELS*, Carl A. PETERS*, Michael KLINE†, Richard E. CUTLER, Jr.*[1], Malathy SHANMUGAM* and Mary HUNZICKER-DUNN*[2]

*Department of Cell and Molecular Biology, Northwestern University Medical School, Chicago, IL 60611, U.S.A., and †Department of Biochemistry, Molecular and Cell Biology, Northwestern University, Evanston, IL 60208, U.S.A.

Small heat-shock proteins (sHSPs) are widely expressed 25–28 kDa proteins whose functions are dynamically regulated by phosphorylation. While recent efforts have clearly delineated a stress-responsive p38 mitogen-activated protein-kinase (MAPK)-dependent kinase pathway culminating in activation of the heat-shock (HSP)-kinases, mitogen-activated protein-kinase-activated protein kinase-2 and -3, not all sHSP phosphorylation events can be explained by the p38 MAPK-dependent pathway. The contribution of protein kinase C (PKC) to sHSP phosphorylation was suggested by early studies but later questioned on the basis of the reported poor ability of purified PKC to phosphorylate sHSP *in vitro*. The current study re-evaluates the role of PKC in sHSP phosphorylation in the light of the isoform complexity of the PKC family. We evaluated the sHSP phosphorylation status in rat corpora lutea obtained from two stages of pregnancy, mid-pregnancy and late-pregnancy, which express different levels of the novel PKC isoform, PKC-δ. Two-dimensional Western blot analysis showed that HSP-27 was more highly phosphorylated *in vivo* in corpora lutea of late pregnancy, corresponding to the developmental stage in which PKC-δ is abundant and active. Late-pregnant luteal extracts contained a lipid-sensitive HSP-kinase activity which exactly co-purified with PKC-δ using hydroxyapatite and S-Sepharose column chromatography. To determine whether there might be preferential phosphorylation of sHSP by a particular PKC isoform, purified recombinant PKC isoforms corresponding to those PKC isoforms detected in rat corpora lutea were evaluated for HSP-kinase activity *in vitro*. Recombinant PKC-δ effectively catalysed the phosphorylation of sHSP *in vitro*, and PKC-α was 30–50 % as effective as an HSP-kinase; other PKCs tested (β1, β2, ϵ and ζ) were poor HSP-kinases. These results show that select PKC family members can function as direct HSP-kinases *in vitro*. Moreover, the observation of enhanced luteal HSP-27 phosphorylation *in vivo*, in late pregnancy, when PKC-δ is abundant and active, suggests that select PKC family members contribute to sHSP phosphorylation events *in vivo*.

INTRODUCTION

The protein kinase C (PKC) family comprises a group of related lipid-sensitive serine/threonine kinase isoforms which differ in regulatory properties, tissue distribution and substrate preferences [1]. The PKC family is broadly divided into three subfamilies based on regulatory properties. The conventional isoforms possess Ca^{2+} and diacylglycerol/phorbol ester sensitivity, the novel isoforms are Ca^{2+}-independent but demonstrate diacylglycerol/phorbol ester sensitivity; the atypical isoforms are insensitive to diacylglycerol/phorbol ester. We have previously investigated the pattern of PKC-isoform expression in the rat ovary as a function of physiological stage. The complement of PKC isoforms identified in rat ovarian tissues includes the conventional PKC-α and PKC-β, the atypical PKC-ζ and the novel PKC-δ [2] and PKC-ϵ isoforms (C. A. Peters, unpublished work). Among these isoforms, the novel PKC-δ isoform is specifically and prominently induced at the end of pregnancy in the rat corpus luteum [2,3]. The present study grew out of our interest to identify luteal proteins which might serve as physiological substrates for the abundant and activated PKC-δ in the corpus luteum of late pregnancy. We have assessed the ability of the small heat-shock protein (sHSP), variously called heat shock protein (HSP)-25 (murine), HSP-27 (rat or human) or HSP-28 (human), to serve as a substrate for PKC-δ.

The sHSPs are widely expressed 25–28 kDa proteins subject to dynamic regulation in response to cell stress, and to specific mitogenic or differentiative stimuli. The dynamic regulation of sHSP includes alterations in amount [4,5], phosphorylation state [6–8], quaternary structure [6] and subcellular localization [9]. Functionally, alterations in sHSP content/phosphorylation result in modulation of actin filament formation and stability [8], growth [10], glutathione metabolism [11] and secretion [9]. Since many of the functions decribed for sHSP are contingent on its ability to be phosphorylated, the complete description of the components of sHSP phosphorylation pathways is critical to a full understanding of sHSP function.

The phosphorylation of sHSPs has been studied both *in vivo* and *in vitro*. Two distinct pathways leading to sHSP phosphorylation *in vivo* can be defined by pharmacological treatment of cells. 'Pathway A', activated in response to stress/heat shock/cytokine treatment of cells, is insensitive to chronic phorbol-ester-induced down-regulation of PKCs, as well as to PKC inhibitors [7,12,13]. Two closely related p38 mitogen-activated protein kinase (MAPK)-activated kinases, mitogen-activated protein kinase-activated protein kinase (MAPKAPK)-2 and MAPKAPK-3, have emerged as the HSP-kinases involved in 'pathway A' phosphorylation of sHSP [14–18].

In contrast to 'pathway A', acute phorbol-ester-induced phosphorylation of sHSP *in vivo* ('pathway B') is blocked by chronic phorbol-ester-induced down-regulation of PKCs and by PKC inhibitors [7,13]. PKC has been implicated as the HSP-

Abbreviations used: PKC, protein kinase C; MAPKAPK, mitogen-activated protein kinase-activated protein kinase; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; eEF, elongation factor; sHSP, small heat-shock protein; HSP, heat-shock protein; MKK, mitogen-activated protein kinase kinase; DTT, dithiothreitol.

[1] Current address: SUGEN Inc., 351 Galveston Drive, Redwood City, CA 94063-4720, U.S.A.

[2] To whom correspondence should be addressed.

kinase in 'pathway B' phosphorylation of sHSP [7,13,19] but the ability to detect direct phosphorylation of sHSP by PKC *in vitro* has varied among laboratories [19–21], resulting in controversy as to whether PKC was indeed an HSP-kinase. This controversy suggested to us that an isoform-selective phosphorylation might be occurring. The present studies show that the Ca^{2+}-independent PKC-δ is superior in its ability to phosphorylate sHSP compared with a panel of other PKC isoforms *in vitro*. This observation is analogous to the ability of the elongation factor (eEF)-1α to serve as a substrate for PKC-δ but not for other PKCs [22]. Furthermore, the detection of phosphorylated sHSP isolated from an intact tissue (the rat corpus luteum of late pregnancy), in which PKC-δ is both abundant and activated, suggests that this phosphorylation has physiological relevance.

MATERIALS AND METHODS

Materials

[γ-^{32}P]ATP (1000–3000 Ci/mmol) was from New England Nuclear; purified recombinant human PKC isoforms were from PanVera Corp., Madison, WI, U.S.A.; purified recombinant murine HSP-25 (recombinant HSP-25) was from Stressgen Biotechnology, Victoria, BC, Canada; purified rabbit skeletal muscle MAPKAPK-2 from Upstate Biotechnology, Lake Placid, NY, U.S.A. and GF109203X was from Alexis, San Diego, CA, U.S.A. 1-Chloro-4-phenyl-3-L-toluene-*p*-sulphonamidobutan-2-one (TPCK) trypsin was from Worthington, histone H1 was from Boehringer–Mannheim, Indianapolis, IN, U.S.A. and electrophoresis reagents were from Bio-Rad, Richmond, CA, U.S.A. Monoclonal HSP-27 antibody was from Dr. Michael Welsh (University of Michigan, Ann Arbor, MI, U.S.A.; [23]), PKC-δ antibody [24] was from Dr. K. Mizuno and Dr. S. Ohno (Yokohama City University School of Medicine, Japan; see Figure 3 below); monoclonal PKC-δ antibody (lot 2, released 4/95) (see Figure 2 below) was from Transduction Laboratories, Lexington, KY, U.S.A.; control and phospho-specific (recognizing phosphotyrosine-182) p38 MAPK antibodies were from New England Biologicals, Beverly, MA, U.S.A. Other chemicals were obtained from Sigma Chemicals, St. Louis, MO, U.S.A.

Animals

Sprague–Dawley rats (Charles River, Portage, MI, U.S.A.) were housed at Northwestern University animal care facilities, maintained in accordance with the 'Guidelines for the Care and Use of Laboratory Animals' by protocols approved by the Northwestern University Animal Care and Use Committee.

Tissue extract preparation

Corpora lutea were dissected from ovaries obtained from mid-pregnant (day 11) or late-pregnant (days 16–22) rats. Dissected tissues were homogenized in either TMES buffer [10 mM Tris/HCl (pH 7.5)/4 mM $MgCl_2$/1.0 mM EGTA/0.32 M sucrose] or in protease/phosphatase inhibitor (PI) buffer, as described by Das et al. [25]. A supernatant fraction (soluble extract) was obtained by centrifuging the homogenate at 105000 ***g*** for 60 min at 4 °C. When indicated, tissues were homogenized in lysis buffer as described by Das et al. [25]. A clarified lysate containing both soluble proteins and detergent-solubilized membrane proteins was obtained by centrifugation of the homogenate at 12000 ***g*** for 10 min at 4 °C.

Phosphorylation incubations and kinase assays

Standard phosphorylation [24] using soluble extracts, column fraction aliquots or recombinant PKC-δ (see Figure 5 below) as enzyme sources was carried out in a final volume of 110 μl, which contained 50 μl of enzyme source, 45 μM α-glycerol phosphate buffer (pH 7.0), 0.9 mM dithiothreitol (DTT), 9.0 mM $MgCl_2$, 4.5 μM ATP and additions as indicated below. Phosphorylations were performed with or without (see Figure 1B) preincubation for 3 min at 37 °C; the reactions were initiated by the addition of 5 μCi [γ-^{32}P]ATP in the presence or absence of the test substances. The test substances added included lipids [phosphatidylserine (45 μg/ml) and 1,2-diolein (1.6 μg/ml)] and either $CaCl_2$ [0.45 mM or 0.9 mM (see Figure 3E)] or EGTA (0.5 mM final concentration). The Ca^{2+}/EGTA buffer system was as described previously [26]. Some incubations contained exogenous substrates [unless otherwise indicated, recombinant HSP-25 or histone H1 at 5 μg/110 μl of reaction medium or recombinant HSP-25 at 2 μg/110 μl of reaction medium (see Figure 3)]. Incubations were performed at 37 °C for 1 min, and were terminated by the addition of 50 μl of 3 × Laemmli SDS stop solution [27] and by heat denaturation at 100 °C for 5 min.

Phosphorylation of recombinant HSP-25 or histone H1 by recombinant PKC isoforms or by purified MAPKAPK-2 (see Figures 4 and 6) included the following modifications. Phosphorylation reactions were carried out, without preincubation, with 20 μM [γ-^{32}P]ATP (6.3 μCi/110 μl of reaction medium) for 3 min. An activity unit was defined as nmol ^{32}P transferred to the substrate/min.

The immune-complex kinase assay [27] was performed on luteal PKC-δ immune complexes prepared by immunoprecipitation. Luteal lysates (400 μg protein) were incubated at 4 °C overnight with 1 μg of monoclonal PKC-δ antibody, followed by incubation for 2 h with 30 μl of Protein A–G-Sepharose (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.). Immune complexes were collected, washed twice with low-salt radioimmunoprecipitation assay (RIPA) buffer [25], once with high-salt RIPA buffer [25] and once with TS buffer [25 mM Tris/HCl (pH 7.0)/50 mM NaCl/200 μM sodium orthovanadate/1 mM DTT] and were finally resuspended in 100 μl of TS buffer. Phosphorylation reactions, using 55 μl of resuspended immune complex as enzyme source, were carried out in an incubation volume of 110 μl containing 40 mM Hepes, pH 7.0, 10 mM $MgCl_2$, 1 mM DTT, 200 μM sodium orthovanadate and 50 μM [γ-^{32}P]ATP (5 μCi/110 μl of reaction medium), 2.5% DMSO and 5 μg of recombinant HSP-25. Where indicated, phosphorylation reaction medium contained the PKC inhibitor, GF109203X [13] (5 μM, final concentration). Phosphorylation reactions were incubated for 15 min at 37 °C and terminated by the addition of 50 μl 3 × Laemmli SDS stop solution and by heat denaturation.

Chromatography

A soluble extract (26 mg protein in TMES buffer) was prepared from luteal tissue (0.65 g wet weight) dissected from 30 late-pregnant rat ovaries. For DEAE–hydroxyapatite chromatography, the extract was applied to a 6-ml column of Whatman DE 52 DEAE cellulose and eluted with a 60-ml linear gradient of 0–300 mM NaCl in TE buffer [10 mM Tris/HCl (pH 7.5)/0.1 mM EGTA], 0.7 ml fractions were collected. The DEAE-resolved PKC peak fractions were pooled and applied to a 3-ml hydroxyapatite column. The column was washed with three column volumes of 40 mM potassium phosphate (pH 7.5)/0.1 mM EGTA, re-equilibrated with 10 mM potassium phosphate and eluted with a 100-ml linear potassium phosphate gradient (10 mM–1.0 M (pH 7.5)/0.1 mM EGTA); 1.0 ml fractions were collected. For S-Sepharose chromatography, the luteal extract was applied to a 2-ml column of S-Sepharose fast flow and was eluted with a 60-ml linear NaCl gradient (0–300 mM in TE buffer); 0.7 ml fractions were collected.

Gel electrophoresis

Separation of luteal proteins from mid-pregnant or late-pregnant ovaries for Western blotting or [^{32}P]phosphoproteins for autoradiography was by SDS/PAGE using 10 % or 12 % separating gels. In some experiments, the lysates were subjected to two-dimensional PAGE, with isoelectric focusing using mixed ampholines (pH 5–8/pH 3–10, 4:1, v/v) in the first dimension and SDS/PAGE in the second dimension [28]. Quantification of [^{32}P]phosphate incorporation was determined by image analysis (Fujix Bioimaging analyser, BAS 2000), by densitometry of autoradiograms (Bio-Rad Imaging Densitometer, model GS-670) or, in some cases, by gel slice analysis. Excised slices were solubilized in 20 % (v/v) H_2O_2 and 20 % (v/v) perchloric acid at 75 °C for 3 h and ^{32}P in the solute was measured by liquid scintillation spectrometry.

Tryptic phosphopeptide analysis

Following phosphorylation of recombinant HSP-25 by PKC-δ or MAPKAPK-2 and subsequent SDS/PAGE, gel slices corresponding to HSP-25 were excised from dried gels for digestion by TPCK–trypsin as described by Cotto et al. [29]. Tryptic peptides were separated by electrophoresis in pH 1.9 buffer at 18 mA for 30 min in the first dimension and by chromatography in the second dimension [29]. Resolved ^{32}P-labelled peptides were revealed by image analysis (Fujix Bioimaging analyser, BAS 2000).

Immunoblotting

Immunoblots were probed with ^{125}I-labelled Protein A (Amersham International) [24] or bands were revealed by enhanced chemiluminescence (ECL; Amersham International) and quantified by densitometry. Bands on p38–MAPK immunoblots were revealed by alkaline phosphatase based enhanced chemiluminescence according to the manufacturer's instructions.

RESULTS

Detection of endogenous HSP-kinase activity in luteal extracts

HSP-27 from rodent sources comprises a single gene product, which can be detected by two-dimensional PAGE as three pH variants representing different phosphorylation states of the same protein [30]. The three phosphorylation states correspond to a more basic unphosphorylated protein, a protein containing a single phosphorylated site and a protein containing two phosphorylated sites per molecule, on Ser-15 and Ser-86 [21,31]. Two-dimensional PAGE followed by Western blotting for HSP-27 was performed in order to compare the phosphorylation state of HSP-27 in lysates prepared from rat corpora lutea of mid (day 11) or late pregnancy (day 22, day of parturition). Three pH variants of HSP-27 were detected in corpora lutea of late pregnancy, designated as a, b and c in Figure 1(A), which corresponded to un-, mono- and di-phosphorylated protein respectively. By contrast, only two pH variants (a and b) of HSP-27 were detected in corpora lutea on day 11 of pregnancy, corresponding to unphosphorylated and monophosphorylated HSP-27 respectively. Thus HSP-27 was more highly phosphorylated *in vivo* in late-pregnant compared with mid-pregnant corpora lutea, suggesting that activated HSP-kinase activities were present in the late-pregnant corpus luteum *in vivo*.

In order to confirm the presence of luteal HSP-kinase activity which has undergone activation *in vivo*, a soluble extract was prepared under conditions that preserved the endogenous phos-

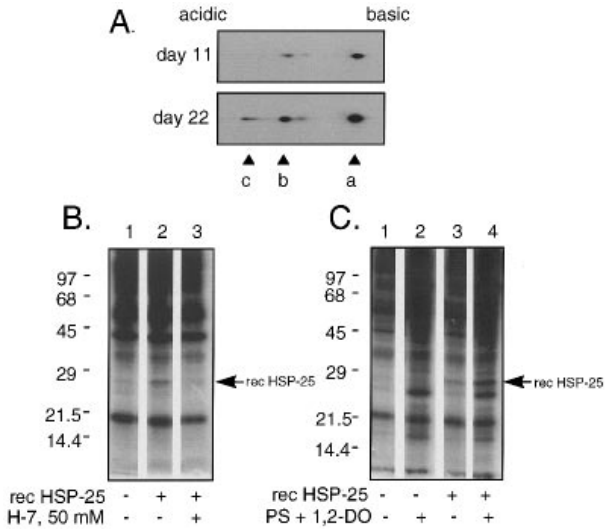


Figure 1 Detection of endogenously phosphorylated sHSP and endogenously activated HSP-kinase activity in luteal preparations

(**A**) Lysates of corpora lutea from days 11 (mid pregnancy) and 22 (late pregnancy, day of parturition) were separated by two-dimensional PAGE with isoelectric focusing using mixed ampholines (pH 5–8) in the first dimension, as described in the Materials and methods section, subjected to Western immunoblotting and probed with HSP-27 antibody. Immunodetected HSP-27 pH variants are indicated by arrowheads marked a, b and c respectively. In phosphorylation reactions, late-pregnant luteal extracts, prepared in the presence of phosphatase inhibitors, were incubated (1 min) with recombinant HSP-25 (rec HSP-25) and (**B**) the kinase inhibitor H-7 or (**C**) phosphatidylserine (PS), 1,2-diolein (1,2-DO) or both, as indicated. All reactions were performed in the absence of Ca^{2+} (in the presence of EGTA). Molecular-mass markers are shown on the left.

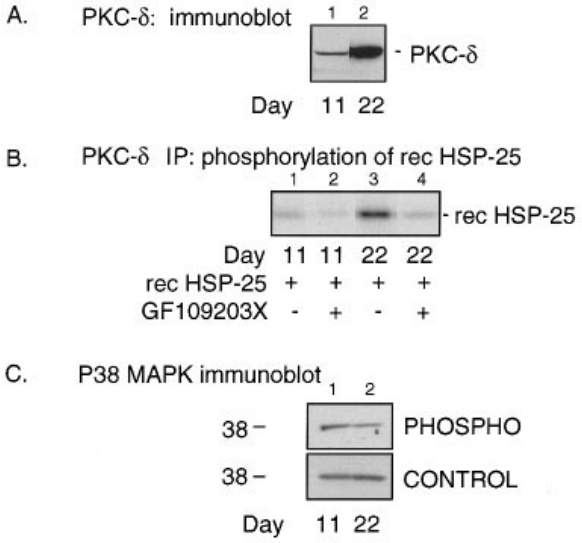


Figure 2 PKC-δ and p38 MAPK kinase activities in corpora lutea of mid (day 11) and late (day 22) pregnancy

(**A**) Western immunoblot of luteal lysates probed with monoclonal PKC-δ antibody. (**B**) PKC-δ kinase activity evaluated by immune complex kinase assay using exogenous recombinant HSP-25 (rec HSP-25) as substrate following specific immunoprecipitation of PKC-δ from luteal lysates. The PKC inhibitor GF109203X was included in control assays, as indicated. (**C**) Western immunoblot of luteal lysates probed with antibody specifically reactive with the activated, phosphorylated form of p38 MAPK (38-) (PHOSPHO) or with antibody reactive with total p38 MAPK (CONTROL).

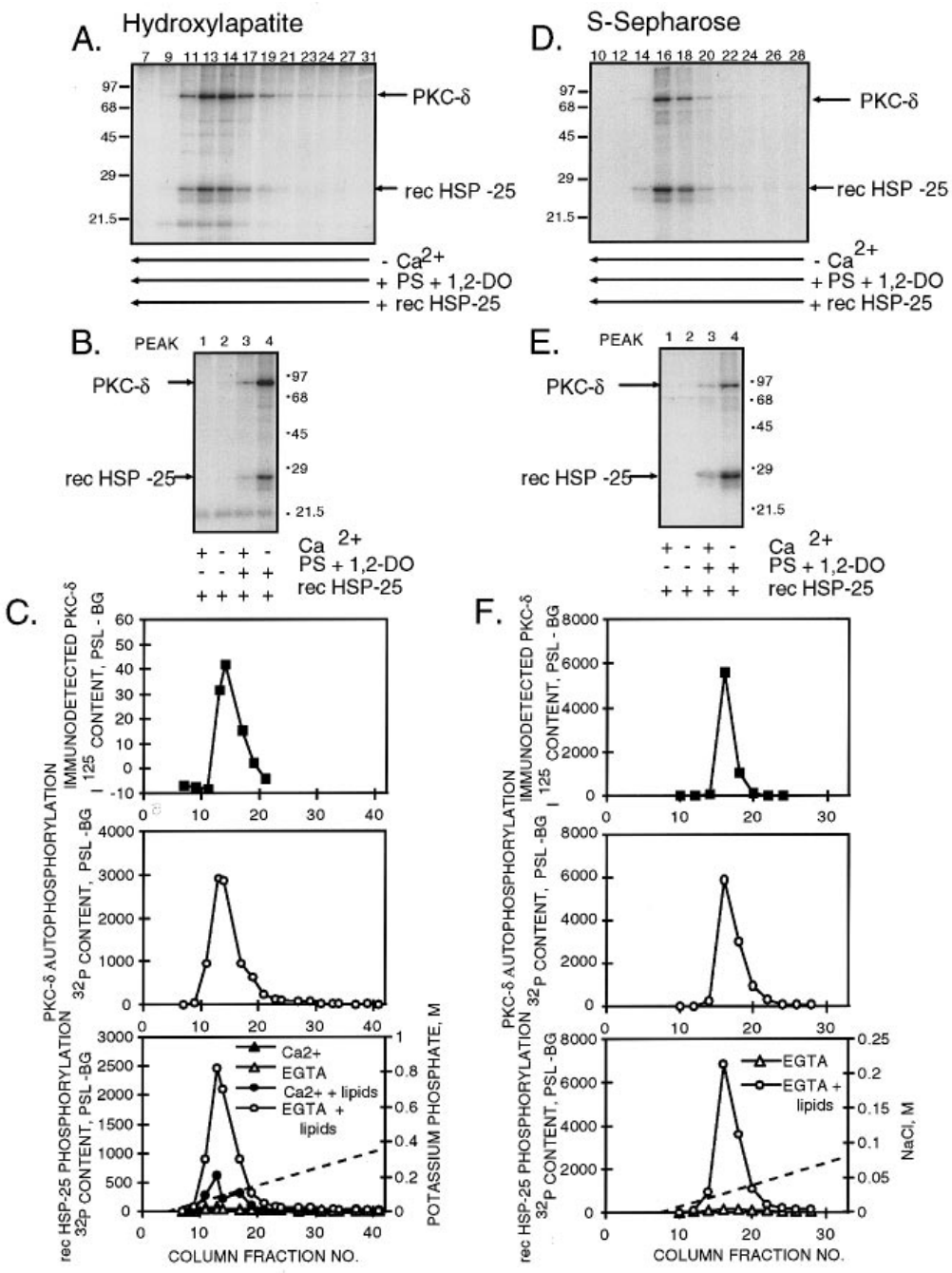
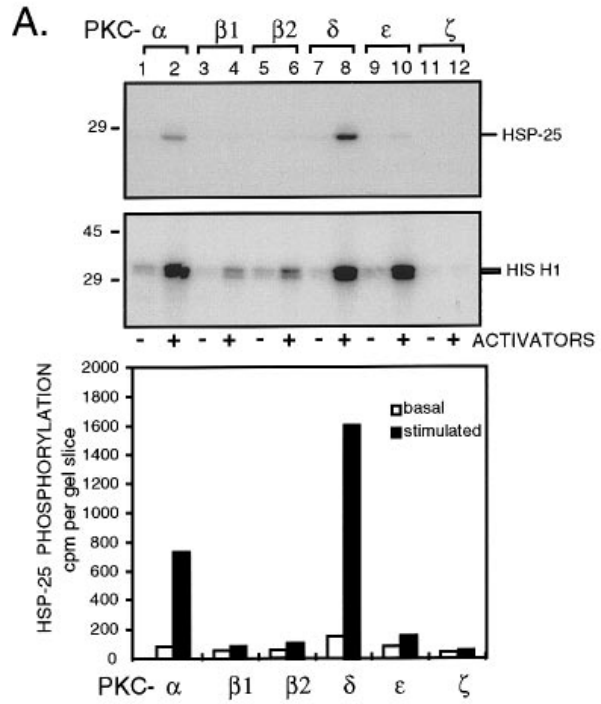


Figure 3 Phosphorylation of HSP-25 by luteal PKC-δ following hydroxyapatite and S-Sepharose chromatography

(**A**—**C**) Analyses of hydroxyapatite fractions. (**D**—**E**) Analyses of S-Sepharose fractions. (**A** and **D**) Autoradiograms of separated [^{32}P]phosphoproteins detected following phosphorylation reactions *in vitro* performed on eluate fractions in the presence of EGTA (— Ca^{2+}), phosphatidylserine (PS) and 1,2-diolein (1,2-DO) and with exogenous recombinant HSP-25 (rec HSP-25) (2 μg/110 μl of reaction medium). The positions of recombinant HSP-25 and of autophosphorylated PKC-δ (identified as described in [2,24]) are indicated by arrows. Molecular-mass markers are shown on the left. (**B** and **E**) Autoradiograms of phosphorylation reactions performed upon the eluted peaks of kinase activity in the presence or absence of activators as indicated [0.45 mM $CaCl_2$ was used in (**B**) and 0.9 mM $CaCl_2$ was used in (**E**)], and in the presence of exogenous recombinant HSP-25 (rec HSP-25). Molecular-mass markers are shown on the right. (**C** and **F**) Immunodetectible PKC-δ (top panels), autophosphorylated PKC-δ (middle panels) and HSP-kinase activity detected by phosphorylation of exogenous recombinant HSP-25 (rec HSP-25) with additions as indicated (bottom panels). Phosphorylations indicated as + lipids included phosphatidylserine and 1,2-diolein. PSL — BG indicates photostimulatable luminescence units minus background.

phorylation state of the kinases (i.e. in the presence of phosphatase inhibitors) and was evaluated for activated HSP-kinase activity. This approach was based on the understanding that the activation state of a number of diverse kinases is linked with their phosphorylation state. As shown in Figure 1(B) (lane 2), the luteal soluble extract contained activated kinase activity which was capable of phosphorylating exogenous recombinant murine HSP-25. This phosphorylation reaction was performed without the addition of exogenous kinase activators, and thus reflects the endogenous activation state of the kinase(s). Incorporation of



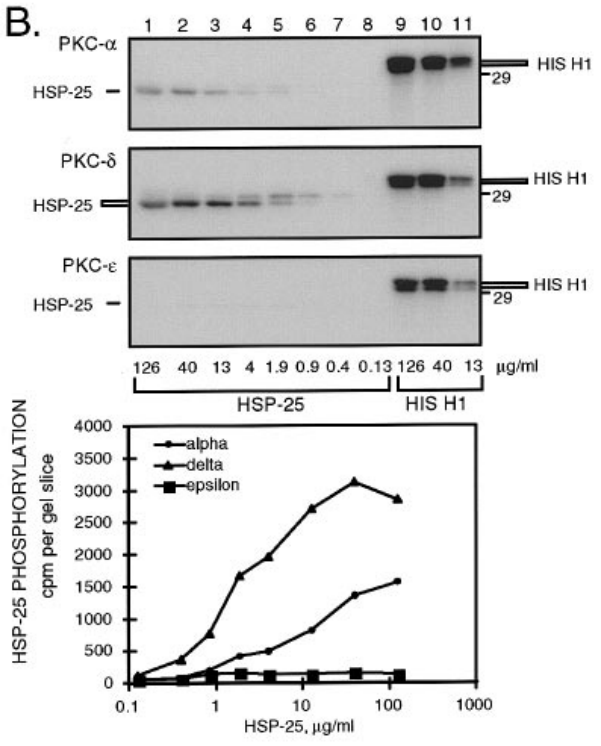


Figure 4 Comparison of HSP-25 phosphorylation by various recombinant PKC isoforms

All incubations were for 3 min with the indicated PKC isoform (3.5 nM) as enzyme source and either recombinant HSP-25 or histone H1 (HIS H1) (45 μg/ml or as indicated) as substrate, and activators were included as indicated. Activators for conventional PKCs (α, β1, β2) were 0.45 mM Ca^{2+}, phosphatidylserine and 1,2-diolein. Activators for novel PKCs (δ, ϵ) were phosphatidylserine and 1,2-diolein, without Ca^{2+} (with EGTA). The activator for atypical PKC (ζ) was phosphatidylserine [53] without Ca^{2+} (with EGTA). All reactions shown in (**B**) contained activators. Phosphorylated recombinant HSP-25 migrated in some instances as a doublet (see **B**), the upper band may represent a phosphorylation-dependent mobility shift.

^{32}P into exogenous recombinant murine HSP-25 in the phosphorylation reaction *in vitro* was decreased by inclusion of the kinase inhibitor H-7 (Figure 1B, lane 3). As H-7 does not inhibit MAPKAPK-2 type HSP-kinase activity [20,32], the decrease in HSP-25 phosphorylation observed indicated that another activated HSP-kinase activity, an H-7-sensitive kinase, was present in the luteal extracts. H-7 is an isoquinolinesulphonamide derivative shown to be a potent and somewhat selective inhibitor for PKC [33], which suggested that the activated kinase could correspond to a PKC isoform.

Phosphorylations of luteal soluble extracts were also performed in the presence of exogenous PKC activators, phosphatidylserine and 1,2-diolein, and in the absence of Ca^{2+}, conditions chosen to specifically enhance the activity of novel (Ca^{2+}-independent) PKC isoforms (Figure 1C). The results showed that further enhancement of recombinant murine HSP-25 phosphorylation was achieved under these conditions, which promoted the selective activation of novel PKC isoforms (Figure 1C, lane 4). In contrast, phosphorylation of HSP-25 was not enhanced by inclusion of lipid activators in the presence of Ca^{2+}, conditions which would support activity of the conventional Ca^{2+}-dependent PKC isoforms (results not shown). As PKC-δ is the dominant novel PKC isoform identified in rat luteal tissue [2,3], the potential involvement of PKC-δ as an HSP-kinase in luteal tissue was further evaluated.

PKC-δ and p38 MAPK activities in mid- versus late-pregnant luteal lysates

Luteal sHSP phosphorylation, as outlined in the Introduction, would be expected to occur through the p38 MAPK-dependent kinase pathway (pathway A) or through a PKC-dependent pathway (pathway B). Kinases representative of these distinct pathways were probed in mid- (day 11) or late- (day 22) pregnant luteal lysates in order to define which pathway might be differentially activated at the end of pregnancy.

We first confirmed the status of PKC-δ in mid- or late-pregnant luteal lysates by Western immunoblotting, using isoform-specific PKC-δ antibody. In agreement with our previous observations on luteal soluble extracts [2,3], PKC-δ protein was induced at the end of pregnancy (Figure 2A). Densitometry of the bands gave a value of 3.84 arbitrary units at day 11 and 21.4 arbitrary units at day 22. The ratio of the densities for day 22/day 11 was 5.6. Levels of other rat luteal PKC isoforms, PKC-α, β1 and β2, ζ and ϵ were not modulated during pregnancy ([3] and C. A. Peters, unpublished work).

The activation state of PKC-δ was assessed by immune-complex kinase assay, and, as shown in Figure 2(B), more active PKC-δ was immunoprecipitated from luteal lysates obtained on day 22 (lane 3) compared with those obtained on day 11 (lane 1) of pregnancy. Densitometry of the bands gave a value of 0.93 arbitrary units for day 11 (lane 1 minus lane 2) and 5.0 units for day 22 (lane 3 minus lane 4). The ratio of the densities for day 22/day 11 was 5.4. The detection of active PKC-δ in luteal lysates at day 22 is consistent with the observed translocation of luteal PKC-δ to a membrane fraction in late pregnancy (C. A. Peters, unpublished work). Thus by immunoblot analysis and by activity assay, PKC-δ was both more abundant and active in day 22 luteal lysates compared with those of day 11, and would be poised to mediate the differential phosphorylation of HSP-27 observed at the end of pregnancy.

The activation state of p38 MAPK at the end of pregnancy was probed by Western blotting with phospho-specific p38 MAPK antibody. As seen in Figure 2(C), the active phos-

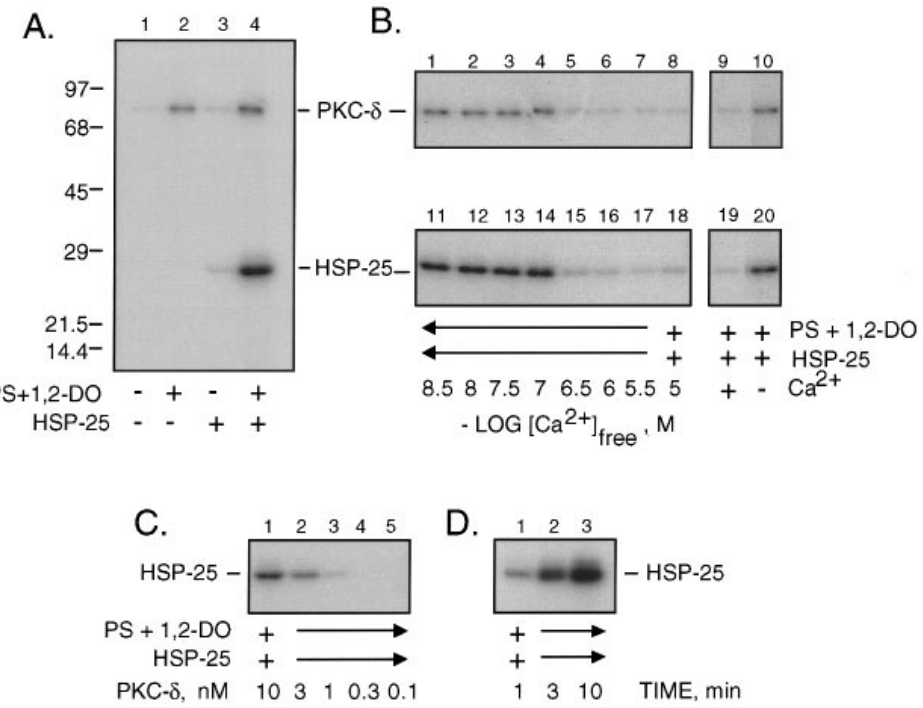


Figure 5 Phosphorylation of HSP-25 by recombinant PKC-δ

Reactions were performed with recombinant PKC-δ (10 nM) as enzyme source, recombinant HSP-25 (45 μg/ml) as substrate and in the absence of Ca^{2+} (presence of EGTA) for 1 min, unless otherwise indicated. (**A**) Phosphorylations were performed in the presence or absence of recombinant murine HSP-25 and in the presence or absence of lipid activators, phosphatidylserine (PS) and 1,2-diolein (1,2-DO), as indicated. Molecular-mass markers are shown on the left. (**B**) To delineate Ca^{2+} inhibition of PKC-δ autophosphorylation (upper panel) and HSP-25 phosphorylation (lower panel), phosphorylation reactions were performed in the presence of Ca^{2+}/EGTA buffer (lanes 1–8 and 11–18) or in the presence of 0.45 mM $CaCl_2$ (lanes 9 and 19) or 1.0 mM EGTA (lanes 10 and 20). (**C**) Phosphorylation reactions were performed in the presence of the indicated final concentrations of PKC-δ. (**D**) Phosphorylation reactions were performed for the indicated times.

phorylated form of p38 MAPK was detected in luteal lysates, however, the amount of active, phosphorylated p38 MAPK was not increased at day 22 when compared with day 11. In contrast to PKC-δ, components of the p38 MAPK pathway would not be set to mediate the differential phosphorylation of HSP-27 observed in day 22 versus day 11 luteal lysates.

Assessment of HSP-kinase activity following resolution of PKC-δ from luteal soluble proteins by chromatography

In order to probe the identity of luteal lipid-stimulated HSP-kinase activity further, resolution of luteal soluble proteins was performed using chromatography. The soluble proteins were initially separated using DEAE-cellulose and the PKC peak fractions were pooled and subjected to further separation by hydroxyapatite chromatography (Figures 3A–C). Alternatively, the luteal soluble proteins were separated by chromatography using a cation-exchange resin, S-Sepharose Fast Flow (Figure 3D–F).

The chromatography fractions were assayed for immunoreactive PKC-δ by Western blotting, for lipid-stimulated autophosphorylation of PKC-δ, and for lipid-stimulated HSP-kinase activity in the presence of added exogenous recombinant HSP-25. Lipid-stimulated phosphorylation of the exogenous HSP-25 substrate was readily noted on autoradiograms (Figures 3A, B, D and E). HSP-kinase activity eluted from each column as a single peak, which exactly corresponded to the elution of PKC-δ activity (Figure 3C, F). The phosphorylation of exogenous HSP-25 had a strict requirement for lipid activators (Figures 3B, C, E and F) and was maximal in the absence of Ca^{2+} (Figures 3B, C and E).

Thus we observed that, using distinct chromatographic protocols, luteal lipid-stimulated HSP-kinase activity co-purified with luteal PKC-δ. Because MAPKAPK-2 and -3 require considerably higher salt concentration for them to be eluted from S-Sepharose-type materials ($>$ 0.15 M NaCl) [15,34,35] compared with that required to elute PKC-δ (0.04 M NaCl) (Figure 3F), it would not be expected that MAPKAPKs would co-elute with PKC-δ.

Comparison of PKC-δ with other PKC isoforms as HSP-kinases

In light of our evidence that luteal PKC-δ displays lipid-stimulated HSP-kinase activity in assays *in vitro*, we wished to establish the ability of purified recombinant PKC-δ to function as a direct HSP-kinase *in vitro*. We compared the ability of recombinant PKC-δ with that of other recombinant PKC isoforms to phosphorylate recombinant murine HSP-25. We found that, whereas PKC-δ readily phosphorylated recombinant murine HSP-25, PKC-α phosphorylated the same substrate to an intermediate extent and the other PKCs tested were poor HSP-kinases (Figure 4A). The phosphorylation of HSP-25 was further evaluated as a function of substrate concentration for three of the enzymes, specifically PKC-α, PKC-δ and PKC-ϵ. The results are shown in Figure 4(B). Again, PKC-δ phosphorylation of HSP-25 was approximately two- to three-fold that of PKC-α, and PKC-ϵ was consistently found to be a poor HSP-kinase at all

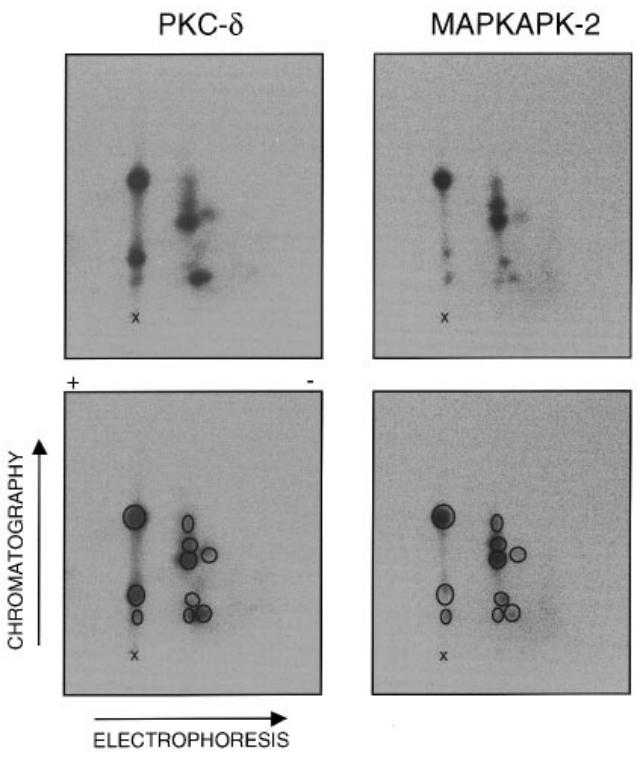


Figure 6 Tryptic phosphopeptide maps of HSP-25 phosphorylated by PKC-δ or MAPKAPK-2

Phosphorylation reactions were performed with either purified MAPKAPK-2 (1.0 units/ml) or recombinant PKC-δ (3.5 nM) as enzyme source and recombinant HSP-25 (45 μg/ml) as substrate. The PKC-δ reaction contained phosphatidylserine and 1,2-diolein. Following SDS/PAGE, ^{32}P-labelled HSP-25 bands were excised, digested and subjected to electrophoresis and chromatography as described in the Materials and methods section. (Top panels) Phosphorimager representations of each map. (Bottom panels) Phosphorimager representations with a common overlay of traced spots superimposed. The origin is indicated as an **X** in the lower left field of each panel.

substrate concentrations tested. Scatchard analysis of the phosphorylation rates for PKC-δ versus PKC-α indicated that PKC-δ had a higher V_{max} for HSP-25 phosphorylation (54.5 units/mg [PKC-δ] versus 20.0 units/mg [PKC-α]): K_m values were similar (112 nM for PKC-δ versus 140 nM for PKC-α). The ratio of V_{max}/K_m, calculated as a measure of efficiency [36], was 482 for PKC-δ and a value of 142 for PKC-α (units·l·mg$^{-1}_{PKC}$·μmol$^{-1}_{HSP\text{-}25}$) respectively.

To further evaluate PKC-δ as a direct HSP-kinase in phosphorylation reactions *in vitro*, additional characteristics of the reaction were probed. HSP-25 was readily phosphorylated by recombinant PKC-δ in a lipid activator- (Figure 5A), enzyme concentration- (Figure 5C) and time- (Figure 5D) dependent manner. It was noted that luteal PKC-δ preparations phosphorylated recombinant HSP-25 most effectively in the absence of Ca^{2+} [see Figures 3B, C (bottom panel) and E]. To assess the effect of Ca^{2+} on the HSP-25 phosphorylation by recombinant PKC-δ, phosphorylation reactions were performed in which free Ca^{2+} concentrations were controlled by the use of a Ca^{2+}/EGTA buffer system. Phosphorylation of HSP-25, as well as autophosphorylation of PKC-δ, was inhibited by increasing concentrations of Ca^{2+} (Figure 5B), a characteristic previously reported for PKC-δ-mediated phosphorylation of GAP-43 [37] and for PKC-δ autophosphorylation [26].

Phosphorylation of HSP-25 by recombinant PKC-δ and MAPKAPK-2: comparison of tryptic phosphopeptide maps

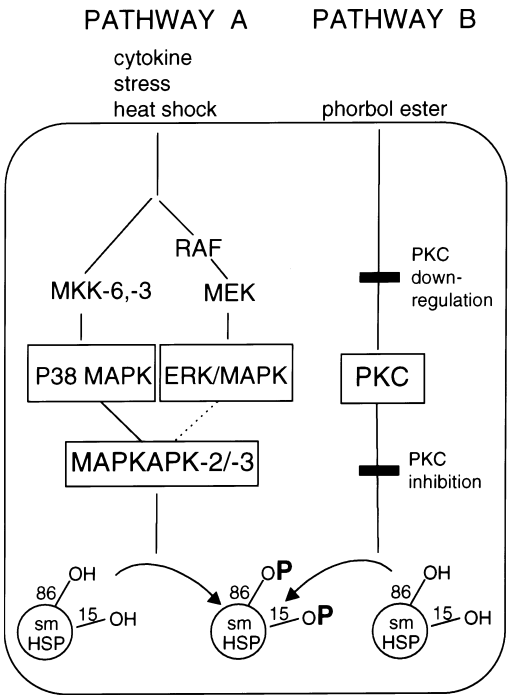
Phosphorylation of rodent HSP-25 on two sites, corresponding to Ser-15 and Ser-86, is amply documented for phosphorylation *in vivo* in response to all classes of stimuli and *in vitro* by the stress-activated kinase MAPKAPK-2 [21,31]. To determine whether the same sites corresponded to sites phosphorylated by PKC-δ, we compared the phosphopeptide maps following tryptic digestion of HSP-25 phosphorylated by PKC-δ and MAPKAPK-2 respectively. As seen in Figure 6, the tryptic-generated phosphopeptide maps are very similar. The arrays of phosphorylated spots on the maps were superimposable, demonstrated by the common traced overlay positioned over each map respectively (Figure 6, bottom panels), although the intensity of certain spots differs between enzymes. The results of tryptic mapping are consistent with the recognition of Ser-15 and Ser-86 as potential phosphorylation sites by PKC-δ, in concert with other enzymes. These results are also consistent with the HSP-25 phosphorylation sites reported for PKC purified without respect to isoform [21]. Notably, the HSP-25 tryptic phosphopeptide maps obtained conformed with the HSP-25 tryptic phosphopeptide map obtained following phosphorylation *in vivo* in intact phorbol-ester-stimulated murine keratinocytes reported previously [38].

DISCUSSION

Our results demonstrated that HSP-27 was detected in a phosphorylated state *in vivo* in the pregnant rat corpus luteum, and was correspondingly more highly phosphorylated at a luteal developmental stage in which PKC-δ was both more abundant and active. Notably, the increased luteal HSP-27 phosphorylation in late pregnancy was not correlated with corresponding changes in p38 MAPK activity. A luteal Ca^{2+}-insensitive, phospholipid/diacylglycerol-sensitive kinase, exactly co-purified with PKC-δ through distinct chromatographic steps and catalysed the phosphorylation of sHSP *in vitro*. Finally, recombinant PKC-δ readily phosphorylated sHSP *in vitro* in a lipid-dependent manner. Recombinant PKC isoforms were compared to determine whether there might be preferential phosphorylation of sHSP by a particular PKC isoform or by a particular subset of PKC isoforms. Recombinant PKC-δ effectively catalysed the phosphorylation of sHSP *in vitro*, whereas PKC-α was 30–50% as effective as an HSP-kinase, and other PKCs tested (β1, β2, ε and ζ) were found to be poor HSP-kinases.

The sHSP exists as a phosphoprotein whose function and structure show dynamic alterations in response to changes in its phosphorylation state. sHSP is phosphorylated on identical serine residues (see below) following exposure of a wide variety of cells to diverse stimuli. Although phosphorylation sites are identical, the susceptibility of sHSP to phosphorylation induced by a particular stimulus or through a particular kinase pathway, is not uniform, but rather it is specific to the cell type and the differentiation state of that cell [5].

The sHSP is subject to phosphorylation in intact cells following exposure to heat shock [13,30], chemical stress [6,8,30], and various cytokines including tumour necrosis factor alpha (TNFα) and interleukin-1 (IL-1) [7,12,30,39]. These diverse treatments have been shown to cause sHSP phosphorylation through the activation of a phosphorylation-dependent kinase cascade schematically depicted as Pathway A in Scheme 1. The pathway A kinase cascade culminates in activation of HSP-kinases, MAPKAPK-2/-3 [14–18]. *In vivo*, p38 MAPKs function as activating kinases immediately upstream of MAPKAPK-2/-3



Scheme 1 Schematic representation of kinase pathways leading to sHSP phosphorylation

For pathway A see review by Denhardt [40], pathway B is based on information in [5,7,13,19,43]. The sHSP (sm HSP) residues subject to phosphorylation are shown as Ser-15 and Ser-86 [21]; these represent identified phosphorylation sites in rodent sHSPs. MKK-6, -3, MAPK kinase types 6 and 3; MEK, MAPK- and ERK-kinase; RAF, Raf protein kinase.

[14,16,17,34,39]; p38 MAPK (the mammalian HOG 1 analogue) is in turn activated by upstream activating kinases, MKK-6 and MKK-3 [40]. The p38 MAPK kinase cascade is analogous to the stress-activated Jun kinase and extracellular signal-regulated kinase (ERK)/MAPK cascades [40]. The sHSP phosphorylation events induced by heat shock/stress/cytokine exposure are resistant to treatments which block PKC, including PKC down-regulation and PKC-specific pharmacological inhibitors [7,12,13], and MAPKAPK-2/-3 activation has been shown to occur independently of PKC activation [17,41,42]. Consistently with these reports, we have recently found follicle-stimulating-hormone induction of sHSP phosphorylation in immature rat ovarian granulosa cells to be correlated with increased p38 MAPK activation, and to be resistant to PKC inhibition (E. T. Maizels, J. Cottom, J. C. R. Jones and M. Hunzicker-Dunn, unpublished work).

Phosphorylation of sHSP has also been found following acute exposure of a variety of cell types to phorbol esters [5,7,13,19,43]. The sensitivity of acute phorbol-ester-stimulated sHSP phosphorylation to PKC down-regulation/inhibition [7,13], in contrast to 'pathway A', supports the notion that phorbol esters cause the phosphorylation of sHSP through a distinct PKC-dependent pathway, depicted in Scheme 1 as 'pathway B'. Moreover, phorbol-ester-stimulated sHSP phosphorylation reactions have been documented to occur without phorbol-ester-induced activation of 'pathway A', at least in some cellular settings. We have found recently phorbol ester stimulation of sHSP phosphorylation in immature rat ovarian granulosa cells to be sensitive to PKC inhibition and to be independent of p38 MAPK (E. T. Maizels, J. Cottom, J. C. R. Jones and M. Hunzicker-Dunn, unpublished work). Likewise, in human lung MRC-5 fibroblasts, phorbol ester treatment induced increased sHSP phosphorylation [12] without inducing activation of the MAPKAPK-2/-3-type HSP-kinase activity [41]. Coexistence of phorbol-ester-stimulated sHSP phosphorylation reactions with heat shock/stress/cytokine-induced sHSP phosphorylation reactions in the same cell type suggests that both pathways A and B could be physiological pathways participating in sHSP phosphorylation in a given cell [7,12,13].

PKC-dependent pathways could feature PKC functioning directly as an HSP-kinase [19] (Scheme 1, pathway B, see also below). PKC might also function indirectly to elicit sHSP phosphorylation through PKC-stimulated activation of other downstream kinases. Phorbol ester treatment can stimulate p38 MAPK activity substantially in some settings [44] but not in others [45,46] (see also [17]). Additionally, the PKC-stimulated activation of the ERK/MAPK pathway has been documented [40]. Although p38 MAPK has been delineated as the upstream activating kinase for MAPKAPK-2/-3 *in vivo* [14,16,34,39], other MAPK families, including ERK/MAPKs, have also been reported to activate MAPKAPK-2/-3 in certain experimental settings [18]. Alternatively PKC could function in a given cell in both direct and indirect modes. We suggest that the relative contributions of direct and indirect modes to overall pathway-B-mediated sHSP phosphorylation could vary, depending on cell type and cell differentiation state and, in particular, depending on which PKC isoforms are present in a cell.

The ability of PKC to function directly as an HSP-kinase has been addressed in several studies *in vitro*, with conflicting findings. Regazzi et al. [19] reported phorbol-ester-stimulated phosphorylation of MCF-7 cell 27-kDa sHSP upon the addition of purified PKC in a reaction *in vitro*. Gaestel et al. [21] observed the phosphorylation of recombinant murine sHSP on two sites by purified PKC *in vitro*. However, Zhou et al. [20] found recombinant hamster sHSP to be a poor substrate for purified PKC *in vitro*. The findings of Zhou et al. [20] have therefore raised questions about the role of PKC as a direct HSP-kinase. However, the isoform composition of the purified PKCs in these studies is not known. The PKC preparation used by Zhou et al. [20] could possibly be comprised of PKC isoforms which do not readily phosphorylate sHSP, and would thus not be representative of the abilities of individual members of the PKC family to serve as HSP-kinases. Our results show that PKC-δ, and to a lesser extent PKC-α, can clearly function as HSP-kinases *in vitro*, whereas the other PKC isoforms analysed are poor HSP-kinases. The findings that phorbol ester could elicit sHSP phosphorylation *in vivo* only in certain cell types and only in certain differentiation states [5], might be explained by differences in the cellular expression of specific PKC isoforms which vary in their ability to function as direct HSP-kinases.

Two major phosphorylation sites, Ser-15 and Ser-86, have been identified in the murine sHSP, HSP-25 [21]. Although the position numbers differ slightly among species, sequences including these sites are conserved in the sHSPs of mammalian species, including the other rodent species. An additional serine residue, representing a minor phosphorylation site in the human HSP-27 (Ser-78), is not conserved in rodent species [30]. Gaestel et al. [21] have postulated that, in murine HSP-25, Ser-15 and Ser-86 are accessible to phosphorylation by various kinases because these sites are structurally exposed. The sequences surrounding the phosphorylation sites (sequence at Ser-15: LLRSPSWE; sequence at Ser-86: LNRQLSSGV) correspond to MAPKAPK-2/-3 recognition sequence 'H'XRXXSXX, where 'H' is a hydrophobic residue [32,34].

It is notable that the sequences surrounding Ser-15 and Ser-86

contain incomplete PKC recognition motifs, specifically RXXS, with a single basic residue proximal to the phosphorylation-site serine residue. A second distal basic residue, which would make these sites correspond to the complete PKC recognition sequence, RXX(S/T)XR, is not found near either site. Recently, a sequence with one or more basic residues proximal to the phosphorylatable serine/threonine residue but having non-basic or hydrophobic residues, rather than basic residues, distal to the phosphorylatable serine/threonine residues [$(R/K)X_{n-1-2}(S/T)XX^*$, where X* is non-basic], has been reported to comprise a PKC-δ preferential phosphorylation sequence [22,47]. Kielbassa et al. [22], studying the phosphorylation of eEF-1α and peptides based on eEF-1α, analysed the sequence preference of PKC-δ compared with other PKC isoforms. These authors found that the presence of a single basic residue proximal to the phosphorylation-site threonine residue in eEF-1α-derived peptides allowed for preferential phosphorylation of that site by PKC-δ when compared with other PKC isoforms. In contrast, the presence of additional basic residues proximal and, most critically, distal to the phosphorylation-site threonine, creating a full PKC recognition site, resulted in loss of selectivity with peptide phosphorylation by all PKC isoforms. Thus the sHSP phosphorylation sites, Ser-15 and Ser-86, lie in PKC-δ-preferential recognition sequences. The similarity of MAPKAPK-2/-3 recognition sequence and PKC-δ preferential recognition sequence suggests that other MAPKAPK-2/-3 substrates, in addition to sHSP, could also potentially be PKC-δ substrates.

Further studies are required to elucidate the function of phosphorylated HSP-27 in the rat corpus luteum of late pregnancy. Phosphorylated HSP-27 is thought to participate in actin filament remodelling, characterized by an increase in filamentous actin at the cell membrane and leading to cell surface blebbing [8], and thus could play a role in luteal hormonal secretory events accompanying parturition. Phosphorylation of sHSP has been associated with secretion in other systems [9] and may drive the accelerated release of relaxin from membrane-bound storage granules in rat luteal cells, resulting in the marked increase in serum relaxin seen in the last three days before parturition [48,49]. Notably, phorbol-ester treatment induced relaxin secretion from porcine luteal cells [50]. Additionally, mediation of actin filament remodelling by phosphorylated HSP-27 could participate in luteal remodelling/regression, i.e. luteolysis, that coincides with parturition. Prostaglandin F2α, a luteolytic agent secreted by the uterus at the end of pregnancy (see [51] for review) has been shown to activate luteal phospholipase C, eliciting phosphatidylinositol turnover and the consequent activation of PKC (see [52] for review).

In summary, the present report shows that HSP-27 exists in rat corpora lutea in a highly phosphorylated state at a time when PKC-δ is both abundant and activated. We have further shown that recombinant HSP-25, the murine analogue of HSP-27, is efficiently phosphorylated by purified PKC-δ and to a lesser extent by PKC-α, whereas other PKC isoforms were not effective HSP-kinases. Recognition of the PKC-isoform selectivity of sHSP phosphorylation may explain and resolve the reported differences among laboratories in the ability to detect direct phosphorylation of sHSP by PKC *in vitro*, and accordingly would help to establish PKC as a 'physiological' HSP-kinase.

This work was supported by National Institute of Child Health and Human Development, grant P01 HD 21921 (to M. H. D.), a Training Program in Reproductive Biology predoctoral fellowship T32 HD 07068 (to C. A. P.), an NCI Training Grant in Carcinogenesis predoctoral fellowship (to M. K.), a Department of Defense Molecular Biology of Breast Neoplasia Training Grant DAMD 17-94-J-4466 (to M. S.) and a Protein Analysis Core of the NIH P30 HD 28048 Center for Reproductive Science grant. We gratefully acknowledge the gifts of PKC-δ antiserum from Dr. S. Ohno and Dr. K. Mizuno (Yokohama City University School of Medicine, Japan) and monoclonal HSP-27 antibody from Dr. Michael Welsh (University of Michigan, Ann Arbor, MI, U.S.A.). We thank Dr. Rasjree M. Rajagopalan-Gupta (Washington State University, Vancouver, WA, U.S.A.) for useful suggestions and Dr. R. I. Morimoto (Northwestern University, Evanston, IL, U.S.A.) for the use of equipment and facilities for tryptic phosphopeptide analysis.

REFERENCES

1 Hug, H. and Sarre, T. F. (1993) Biochem. J. **291**, 329–343
2 Cutler, Jr., R. E., Maizels, E. T., Brooks, E. J., Mizuno, K., Ohno, S. and Hunzicker-Dunn, M. (1993) Biochem. Biophys. Acta **1179**, 260–270
3 Cutler, Jr., R. E., Maizels, E. T. and Hunzicker-Dunn, M. (1994) Endocrinology **135**, 1669–1678
4 Spector, N. L., Ryan, C., Samson, W., LeVine, H., Nadler, L. M. and Arrigo, A. P. (1993) J. Cell. Physiol. **156**, 619–625
5 Minowada, G. and Welch, W. (1995) J. Biol. Chem. **270**, 7047–7054
6 Kato, K., Hasegawa, K., Goto, S. and Inaguma, Y. (1994) J. Biol. Chem. **269**, 11274–11278
7 Arrigo, A. P. (1990) Mol. Cell. Biol. **10**, 1276–1280
8 Landry, J. and Huot, J. (1995) Biochem. Cell Biol. **73**, 703–707
9 Zhu, Y., O'Neill, S., Saklatvala, J., Tassi, L. and Mendelsohn, M. E. (1994) Blood **84**, 3715–3723
10 Kindas-Mugge, I., Herbacek, I., Jantschitsch, C., Micksche, M. and Trautinger, F. (1996) Cell Growth Differ. **7**, 1167–1174
11 Mehlen, P., Kretz-Remy, C., Preville, X. and Arrigo, A.-P. (1996) EMBO J. **15**, 2695–2706
12 Saklatvala, J., Kaur, P. and Guesdon, F. (1991) Biochem. J. **277**, 635–642
13 Faucher, C., Capdevielle, J., Canal, I., Ferrara, P., Mazarguil, H., McGuire, W. L. and Darbon, J. M. (1993) J. Biol. Chem. **268**, 15168–15173
14 Rouse, J., Cohen, P., Trigon, S., Morange, M., Alonso-Llamazares, A., Zamanillo, D., Hunt, T. and Nebreda, A. R. (1994) Cell **78**, 1027–1037
15 Freshney, N. W., Rawlinson, L., Guesdon, F., Jones, E., Cowley, S., Hsuan, J. and Saklatvala, J. (1994) Cell **78**, 1039–1049
16 Guay, J., Lambert, H., Gingras-Breton, G., Lavoie, J. N., Huot, J. and Landry, J. (1997) J. Cell Sci. **110**, 357–368
17 McLaughlin, M. M., Kumar, S., McDonnell, P. C., Van Horn, S., Lee, J. C., Livi, G. P. and Young, P. R. (1996) J. Biol. Chem. **271**, 8488–8492
18 Ludwig, S., Engel, K., Hoffmeyer, A., Sithanandam, G., Neufeld, B., Palm, D., Gaestel, M. and Rapp, U. R. (1996) Mol. Cell. Biol. **16**, 6687–6697
19 Regazzi, R., Eppenberger, U. and Fabbro, D. (1988) Biochem. Biophys. Res. Commun. **152**, 62–68
20 Zhou, M., Lambert, H. and Landry, J. (1993) J. Biol. Chem. **268**, 35–43
21 Gaestel, M., Schroder, W., Benndorf, R., Lippmann, C., Buchner, K., Hucho, F., Erdmann, V. A. and Bielka, H. (1991) J. Biol. Chem. **266**, 14721–14724
22 Kielbassa, K., Muller, H. J., Meyer, H. E., Marks, F. and Gschwendt, M. (1995) J. Biol. Chem. **270**, 6156–6162
23 Smoyer, W. E., Gupta, A., Mundel, P., Ballew, J. D. and Welsh, M. J. (1996) J. Clin. Invest. **97**, 2697–2704
24 Maizels, E. T., Miller, J. B., Cutler, Jr., R. E., Jackiw, V., Carney, E. M., Mizuno, K., Ohno, S. and Hunzicker-Dunn, M. (1992) J. Biol. Chem. **267**, 17061–17068
25 Das, S., Maizels, E. T., DeManno, D., St. Clair, E., Adam, S. A. and Hunzicker-Dunn, M. (1996) Endocrinology **137**, 967–974
26 Maizels, E. T., Miller, J. B., Cutler, Jr., R. E., Jackiw, V., Carney, E. M., Kern, L. and Hunzicker-Dunn, M. (1990) Biochim. Biophys. Acta **1054**, 285–296
27 Shanmugam, M., Krett, N. L., Peters, C. A., Maizels, E. T., Murad, F. M., Kawakatsu, H., Rosen S. T. and Hunzicker-Dunn, M. (1998) Oncogene **16**, 1649–1654
28 Hunzicker-Dunn, M., Cutler, Jr., R. E., Maizels, E. T., DeManno, D. A., Lamm, M. L. G., Erlichman, J., Sanwal, B. D. and LaBarbera, A. R. (1991) J. Biol. Chem. **266**, 7166–7175
29 Cotto, J. J., Kline, M. and Morimoto, R. I. (1996) J. Biol. Chem. **271**, 3355–3358
30 Landry, J., Lambert, H., Zhou, M., Lavoie, J. N., Hickey, E., Weber, L. A. and Anderson, C. W. (1992) J. Biol. Chem. **267**, 794–803
31 Stokoe, D., Engel, K., Campbell, D. G., Cohen, P. and Gaestel, M. (1992) FEBS Lett. **313**, 307–313
32 Stokoe, D., Caudwell, B., Cohen, P. T. and Cohen, P. (1993) Biochem. J. **296**, 843–849
33 Hidaka, H., Watanabe, M. and Kobayashi, R. (1991) Methods Enzymol. **201**, 328–339
34 Clifton, A. D., Young, P. R. and Cohen, P. (1996) FEBS Lett. **392**, 209–214
35 Ahlers, A., Belka, C., Gaestel, M., Lamping, N., Sott, C., Herrmann, F. and Brach, M. A. (1994) Mol. Pharmacol. **46**, 1077–1083
36 Olivier, A. R. and Parker, P. J. (1991) Eur. J. Biochem. **200**, 805–810

37 Dekker, L. V. and Parker, P. J. (1997) J. Biol. Chem. **272**, 12747–12753
38 Kasahara, K., Ikuta, T., Chida, K., Asakura, R. and Kuroki, T. (1993) Eur. J. Biochem. **213**, 1101–1107
39 Foltz, I. N., Lee, J. C., Young, P. R. and Schrader, J. W. (1997) J. Biol. Chem. **272**, 3296–3301
40 Denhardt, D. T. (1996) Biochem. J. **318**, 729–747
41 Guesdon, F., Freshney, N., Waller, R. J., Rawlinson, L. and Saklatvala, J. (1993) J. Biol. Chem. **268**, 4236–4243
42 Cano, E., Doza, Y. N., Ben-Levy, R., Cohen, P. and Mahadevan, L. C. (1996) Oncogene **12**, 805–812
43 Welch, W. J. (1985) J. Biol. Chem. **260**, 3058–3062
44 Wesselborg, S., Bauer, M. K. A., Vogt, M., Schmitz, M. L. and Schulze-Osthoff, K. (1997) J. Biol. Chem. **272**, 12422–12429
45 Jiang, Y., Chen, C., Li, Z., Guo, W., Gegner, J. A., Lin, S. and Han, J. (1996) J. Biol. Chem. **271**, 17920–17926
46 Geng, Y., Valbracht, J. and Lotz, M. (1996) J. Clin. Invest. **98**, 2425–2430
47 Nishikawa, K., Toker, A., Johannes, F.-J., Songyang, Z. and Cantley, L. C. (1997) J. Biol. Chem. **272**, 952–960
48 Sherwood, O. D., Crnekovic, V. E., Gordon, W. L. and Rutherford, J. E. (1980) Endocrinology **107**, 691–698
49 Fields, P. A. (1984) Biol. Reprod. **30**, 753–762
50 Taylor, M. J. and Clark, C. L. (1988) Biol. Reprod. **39**, 743–750
51 Rothchild, I. (1981) Rec. Prog. Horm. Res. **37**, 183–298
52 Niswender, G. D., Juengel, J. L., McGuire, W. J., Belfiore, C. J. and Wiltbank, M. C. (1994) Biol. Reprod. **50**, 239–247
53 Nakanishi, H. and Exton, J. H. (1992) J. Biol. Chem. **267**, 16347–16354

Received 11 December 1997/9 March 1998; accepted 12 March 1998