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Unphosphorylated α-PKC Exhibits Phorbol Ester Binding but Lacks Protein Kinase Activity In Vitro

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Abstract Expression of the α-isoform of protein kinase C (α-PKC) in *E. coli* yielded the unphosphorylated 74 kD precursor molecule. This precursor form exhibited phospholipid- and calcium-dependent phorbol ester binding but lacked, in contrast to the phosphorylated enzyme, protein kinase activity. In addition, the precursor molecule was found to interact with both threonine and an ATP analogon, which demonstrates that phosphorylation of α-PKC is not required for binding of substrates, cofactors, or activators. These results, therefore, suggest that posttranslational phosphorylation of α-PKC is not needed for the formation of a functional enzyme-substrate complex but is necessary for the catalytic transfer of phosphate residues from ATP to protein substrates.

Key words: protein expression in *E. coli*, enzyme activation, posttranslational phosphorylation, α-PKC

Cellular signalling mediated by protein kinase C (PKC), a multigene family, is thought to represent a major control element in cell growth and differentiation [Nishizuka, 1986, 1988]. Stimulation of a broad variety of cell surface receptors by appropriate growth factors, hormones, and neurotransmitters is known to induce activation of PKC via the formation of diacylglycerol and the mobilization of calcium [Nishizuka, 1986, 1988]. Since PKC activation by diacylglycerol has been shown to be mimicked by tumor promoting phorbol esters, its involvement in malignant growth has been suggested [Castagna et al., 1982; Parker et al., 1984].

Besides the regulation of the enzymatic activity by diacylglycerol and phorbol esters, PKC is also regulated by posttranslational modifications. α-PKC is known to be synthesized as a precursor molecule which is subsequently phosphorylated yielding the mature enzyme [Borner et al., 1989; Parker et al., 1987; Patel and Stabel, 1989]. Since the phosphorylated form is found to exhibit protein kinase activity and phorbol ester binding, the hypothesis was made that the unphosphorylated molecule may represent an inactive precursor form [Borner et al., 1989; Pears et al., 1992]. However, the characterization of the precursor molecule was so far not possible due to the lack of its accumulation in eukaryotic cells.

Here we show that expression of α-PKC in bacteria yielded the unphosphorylated precursor which was analyzed with respect to protein kinase and phorbol esters binding activity.

MATERIALS AND METHODS

Materials

λbPKC306 [Parker et al., 1986] was given by P.J. Parker (Ludwig Institute for Cancer Research, London), pET-8c [Studier et al., 1990] by F.W. Studier (Biology Department, Brookhaven National Laboratory, NY), and pING1 [Johnston et al., 1985] by J.H. Lee (INGENE, Santa Monica, CA). LLC-PK51 is a pig kidney cell line which overexpresses bovine α-PKC from the same cDNA as used for the expression in bacteria [Wartmann et al., 1991]. *E. coli* MC1061 was obtained from BIO-RAD and restriction enzymes, Klenow DNA polymerase, and T4 DNA ligase from New England Biolabs. Buffer substances and salts for media were from Merck, carbohydrate supplements and amino acid components from Sigma, anti α-PKC antibody MC5,

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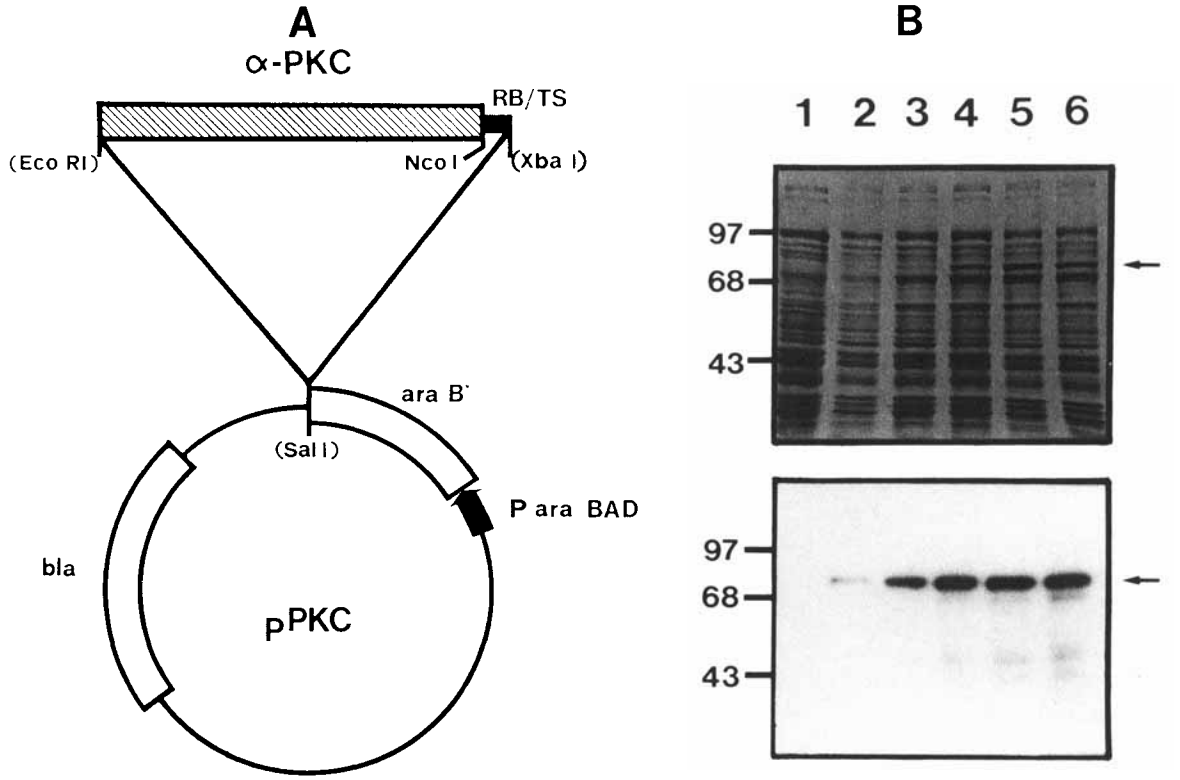

Fig. 1. Expression of α-PKC in *E. coli*. **A:** Construction of the plasmid pPKC. α-PKC cDNA was cloned into the bacterial expression vector pING1. Details are described in Materials and Methods. Restriction sites used are indicated (parentheses mark blunt-ended sites). The arabinose-inducible promoter P araBAD is represented by a filled arrow, the ara B′ and the bla gene (Ap^r) by open boxes. RB/TS stands for the DNA fragment containing a translational stop and a ribosomal binding site. **B:** Expression of α-PKC upon induction by arabinose. Cultures of MC-PKC were grown and α-PKC expression was induced by the addition of arabinose as described. Cell lysates were prepared, subjected to $NaDodSO_4$-PAGE (8% polyacrylamide), and analyzed by either Coomassie brilliant blue staining (**upper panel**) or by immunoblotting (**lower panel**). **Lanes 1–6:** 0, 1, 3, 6, 16, and 24 h after induction, respectively. The arrow marks α-PKC protein. Molecular mass standards (in kD) are shown on the left.

[^{125}I]-labelled antimouse antibody, and protein A-sepharose CL-4B from Amersham, and horse radish peroxidase-coupled antimouse antibody from Boehringer Mannheim. [^{32}P]-orthophosphoric acid and [^{35}S]-methionine were obtained from New England Nuclear. [^{3}H]-phorbol 12,13 dibutyrate ([^{3}H]-PDBu) and [γ^{32}P]-ATP were from Amersham, L-α-phosphatidyl-L-serine, dioelin, Histone HIII, and protamine sulfate were from Sigma, and acrylamide and N, N′-methylenebisacrylamide from Serva.

Expression of α-PKC in *E. coli*

The 2.1 kb cDNA fragment coding for the bovine brain α-PKC was obtained by an EcoRI and a partial NcoI digestion of λbPKC306. A sequence of 39 bp containing a translational stop and a ribosomal binding site (the XbaI/NcoI fragment of pET-8c) was ligated to the isolated 2.1 kb DNA fragment. This extended DNA fragment was blunt-ended by Klenow DNA polymerase and subsequently cloned into the blunt-ended SalI site of the expression vector pING1 yielding the plasmid pPKC (Fig. 1A). pING1 has originally been designed for the expression of target proteins fused to ara B′ sequences. However, the insertion of the additional 39 bp between ara B′ and α-PKC permitted α-PKC to be expressed as a non-fused protein.

E. coli MC1061 was transformed with pPKC by electroporation (thereafter called MC-PKC) and 5 ml cultures were grown in M9 minimal medium [Sambrook et al., 1989] supplemented with 0.4% fructose. At an optical density OD_{600} of 0.5, expression of α-PKC was induced by adding arabinose to a final concentration of 0.2% for indicated times. Cells were pelleted by centrifugation (1 min at 12,000*g*) and disrupted by sonication in 500 μl buffer A (20 mM Tris-HCl pH 7.4, 1 mM EDTA, 1 mM EGTA, and 10 mM

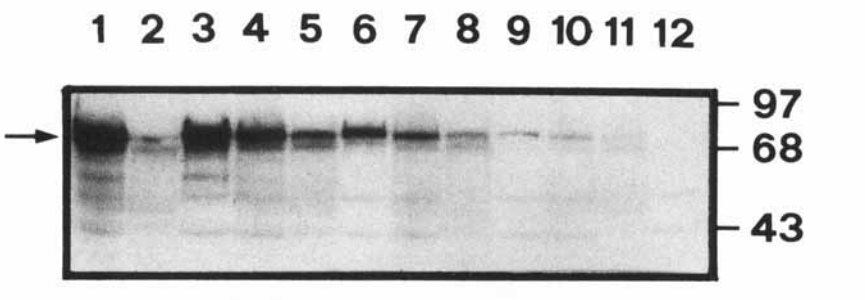

Fig. 2. Immunoblot analysis of the subcellular distribution of α-PKC in *E. coli* Cultures of MC-PKC were grown and induced for 24 h at 37°C (**lanes 1–3**), 30°C (**lanes 4–6**), 25°C (**lanes 7–9**), and 18°C (**lanes 10–12**), respectively Total extracts (lanes 1, 4, 7, 10), fractions of soluble (lanes 2, 5, 8, 11) and insoluble proteins (lanes 3, 6, 9, 12) were prepared and immunoblot analysis was performed The arrow marks the position of α-PKC protein Molecular mass standards (in kD) are shown on the right

2-mercaptoethanol). Finally, "soluble" (supernatants) and "insoluble" (pellets) protein fractions were obtained by high speed centrifugation (30 min at 100,000*g*).

Labelling of α-PKC

Cultures (5 ml) of MC-PKC were grown in a low phosphate, methionine free M9 medium supplemented with 0.4% fructose to an optical density OD_{600} of 0.5. Expression of α-PKC was induced by arabinose and proteins were labelled by the addition of [^{35}S]-methionine (50 μCi/ml) or [^{32}P]-phosphoric acid (50 μCi/ml). Soluble protein fractions were prepared as described above.

Immunological Analysis of α-PKC

Immunoblot analysis and immunoprecipitation of α-PKC have been performed as described [Borner et al., 1988, 1991].

Protein Kinase Activity and [^{3}H]-PDBu Binding of α-PKC

Kinase and [^{3}H]-PDBu binding activity of α-PKC have been measured as described [Regazzi et al., 1986]. The affinity of α-PKC for [^{3}H]-PDBu was determined as described [Costa et al., 1985].

RESULTS

Expression of α-PKC in *E. coli*

In order to express bovine α-PKC in *E. coli*, the expression plasmid pPKC has been constructed (Fig. 1A) and transformed into *E. coli* MC1061, generating the MC-PKC cell. Induction of MC-PKC led to the accumulation of a protein which was identified to be α-PKC (Fig. 1B). Unfortunately, but consistent with previous reports [Marston, 1986, and references therein], nearly all of the expressed α-PKC was found in the insoluble protein fraction (Fig. 2, lanes 1–3). However, relative amounts of soluble α-PKC protein drastically increased after lowering the expression temperature (Fig. 2, lanes 4–12). Expression at 30°C yielded highest levels of soluble protein.

α-PKC Expressed in *E. coli* Represents the Unphosphorylated Precursor Form

Phosphorylation of the PKC precursor yielding the mature enzyme is known to cause a shift of the apparent molecular weight of the molecule from 74 to 77 kD [Young et al., 1987; Parker et al., 1987; Borner et al., 1989; Patel and Stabel, 1989]. In order to detect alterations in the molecular mass of the enzyme, reflecting phosphorylations, the mobility of the protein on SDS polyacrylamide gels was analyzed. MC-PKC was found to generate an α-PKC species exhibiting a smaller molecular size than the phosphorylated 77 kD form present in eukaryotic cells (Fig. 3A). In addition, determining the state of phosphorylation by differential labelling, α-PKC expressed in bacteria was found to incorporate [^{35}S]-methionine but not [^{32}P]-phosphate (Fig. 3B). These results strongly indicate that α-PKC expressed in *E. coli* represents the unphosphorylated 74 kD precursor form of this enzyme.

Binding of Phorbol Ester by the Unphosphorylated α-PKC Precursor

The 74 kD α-PKC precursor was analyzed with respect to phorbol ester binding, an activity that has been demonstrated for most of the members of the PKC family [Niedel et al., 1983; Leach et al., 1983; Parker et al., 1984; Nishi-

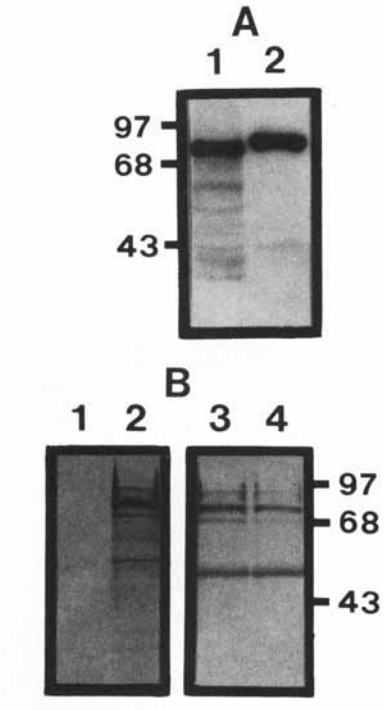

Fig. 3. Characterization of α-PKC expressed in *E coli* **A:** Determination of the molecular mass Total cell extracts from arabinose-induced MC-PKC (**lane 1**) and from LLC-PK51, a pig kidney cell line which overexpresses the same cDNA as MC-PKC [Wartmann et al , 1991] (**lane 2**) were subjected to immunoblot analysis after NaDodSO$_4$-PAGE Molecular mass standards (in kD) are shown on the left **B:** Determination of the phosphorylation state Cultures of MC-PKC were induced and labelled by the addition of [^{32}P]-phosphoric acid (**lanes 1, 3**) or [^{35}S]-methionine (**lanes 2, 4**) Fractions of soluble proteins were prepared and α-PKC was immunoprecipitated Precipitated proteins were separated by NaDodSO$_4$-PAGE and analyzed by either fluorography (lanes 1, 2) or by immunoblotting using a non-radioactive horse radish peroxidase-coupled secondary antibody (lanes 3, 4) The signals at 50 kD in lanes 3 and 4 represent the heavy chain of the antibodies used to precipitate α-PKC, which cross-react with the antibody used for immunoblotting

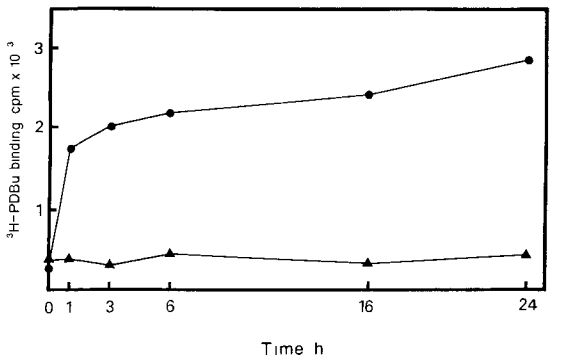

Fig. 4. [^{3}H]-PDBu binding activity in *E coli* extracts Cultures of MC-PKC (dots) and MC-pING1 (triangles) were grown and induced by arabinose Aliquots were taken at 0, 1, 3, 6, 16, and 24 h after induction, extracts of total proteins were prepared, and [^{3}H]-PDBu binding was measured The values indicated are the means of three independent experiments

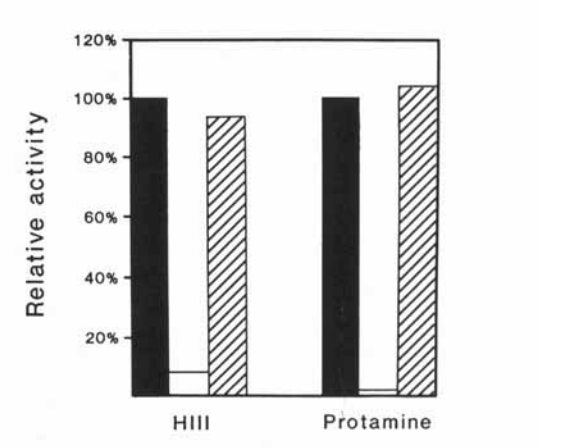

Fig. 5. Kinase activity in *E coli* extracts Purified α-PKC from eukaryotic LLC-PK51 (filled bars), from bacterial MC-PKC (open bars), and a mixture of both (hatched bars) were assayed for histone HIII and protamine sulfate phosphorylations α-PKC concentrations were adjusted by immunoblot prior to the assay Results represent the means of three independent experiments and were normalized to the activities of α-PKC from LLC-PK51

zuka, 1988] (Fig. 4). In contrast to protein extracts of the vector control MC-pING 1, extracts of MC-PKC were found to bind radiolabelled phorbol 12,13 dibutyrate ([^{3}H]-PDBu) upon induction. The extent of binding correlated with α-PKC levels was determined by immunoblot analysis (Fig. 1B). [^{3}H]-PDBu binding to MC-PKC extracts was strictly dependent on phosphatidyl serine (PS) and calcium. Scatchard analysis indicated that the molecule binds PDBu with an affinity of 9 ± 2 nM (data not shown). Therefore, binding of the cofactors calcium and PS as well as of the activating phorbol esters is not dependent on phosphorylation of the α-PKC molecule.

Unphosphorylated α-PKC Precursor Features No In Vitro Kinase Activity

The 74 kD α-PKC precursor was partially purified from soluble extracts of MC-PKC by two chromatographic steps using threonine sepharose and the ATP-analogon Cibacron Blue F3G-A (data not shown). The enzyme was then analyzed with respect to histone HIII and protamine sulfate phosphorylation and compared to α-PKC purified by the same procedure from the eukaryotic LLC-PK51 cell line [Wartmann et al., 1991] (Fig. 5). In contrast to phosphorylated α-PKC from LLC-PK51, unphosphorylated

α-PKC from MC-PKC exhibited no in vitro kinase activity. The possible presence of an inhibitor co-purified with α-PKC from the bacterial lysate could be excluded since no inhibitory effect was observed when a mixture of both preparations was assayed (Fig. 5). These results indicate that the unphosphorylated 74 kD α-PKC precursor lacks the potential to phosphorylate known PKC substrates.

DISCUSSION

By expressing α-PKC in *E. coli* we succeeded in producing the unphosphorylated 74 kD precursor molecule. Isolation of the α-PKC precursor has not been possible so far due to its rapid modification in eukaryotic cells. The characterization of this precursor protein is of interest since the unphosphorylated 74 kD molecule has been postulated to represent the inactive precursor form of α-PKC which is converted to the active enzyme by a specific phosphorylation [Borner et al., 1989; Pears et al., 1992].

The unphosphorylated 74 kD α-PKC precursor molecule was found to bind the phorbol ester PDBu in a calcium- and phospholipid-dependent manner. Zinc-finger structures within the conserved region C1 of the enzyme have been reported to be responsible for the phospholipid-dependent binding of phorbol esters [Ono et al., 1989; Cazaubon et al., 1990; Burns and Bell, 1991]. In addition, mutations within the zinc-fingers have been shown to lead to a total loss of PDBu binding, indicating that an intact secondary structure of PKC is essential for binding of activators [Ono et al., 1989]. In accordance with these reports our result suggests that the unphosphorylated 74 kD precursor protein possesses native zinc-finger structures as well as a functional C2 domain, the calcium binding motif of cPKCs [Kaibuchi et al., 1989; Ono et al., 1989]. Furthermore, our finding demonstrates that posttranslational phosphorylation of α-PKC is neither needed for binding of cofactors and activators nor for the formation of protein structures appropriate for these binding activities.

The failing of the unphosphorylated 74 kD α-PKC to phosphorylate substrate proteins confirmed the postulated lack of kinase activity of this precursor molecule. An incorrect folding of the enzyme has been considered to be a possible reason for the missing kinase activity. However, the presence of functional C1, C2, C3, and C4 domains reflected by the interactions of the α-PKC precursor with activators (PDBu), cofactors (calcium and PS), and substrates (threonine and the ATP-analogon) point to a correct enzyme structure. Therefore, we propose that the unphosphorylated state of the 74 kD α-PKC precursor is responsible for the missing kinase activity of this protein form.

Summarizing, our results indicate that phosphorylation of α-PKC is not required for the binding of effectors, activators, or substrates, but is necessary for protein kinase activity. We conclude that posttranslational phosphorylation of α-PKC is not involved in the formation of the functional complex consisting of the enzyme, activators, and substrates but is needed for catalyzing the subsequent transfer of phosphate residues from ATP to protein substrates.

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REFERENCES

Borner C, Eppenberger U, Wyss R, Fabbro D (1988) Continuous synthesis of two protein kinase C-related proteins after down-regulation by phorbol esters Proc Natl Acad Sci USA 85 2110–2114

Borner C, Filipuzzi I, Wartmann M, Eppenberger U, Fabbro D (1989) Biosynthesis and posttranslational modifications of protein kinase C in human breast cancer cells J Biol Chem 264 13902–13909

Borner C, Filipuzzi I, Weinstein IB, Imber R (1991) Failure of wild-type or mutant form of protein kinase C-α to transform fibroblasts Nature 353 78–80

Burns DJ, Bell RM (1991) Protein kinase C contains two phorbol ester binding domains J Biol Chem 266 18330–18338

Castagna M, Takai Y, Kaibuchi K, Sano K, Kikkawa U, Nishizuka Y (1982) Direct activation of calcium-activated, phospholipid-dependent protein kinase by tumor promoting phorbol esters J Biol Chem 257 7847–7851

Cazaubon S, Webster C, Camoin L, Strosberg DA, Parker PJ (1990) Effector-dependent conformational changes in protein kinase Cγ trough epitope mapping with inhibitory monoclonal antibodies Eur J Biochem 194 799–804

Costa SD, Fabbro D, Regazzi R, Kung W, Eppenberger U (1985) The phorboid receptor correlates with hormone dependency in six mammary carcinoma cell lines Biochem Biophys Res Commun 133 814–822

Johnston SJ, Lee J-H, Ray DS (1985) High-level expression of M13 gene II protein from an inducible polycistronic messenger RNA Gene 34 137–145

Kaibuchi K, Fukumoto Y, Oku N, Takai Y, Arai K-I, Muramatsu MA (1989) Molecular genetic analysis of the regulation and catalytic domains of protein kinase C J Biol Chem 264 13489–13496

Leach KL, James ML, Blumberg PM (1983) Characterization of a specific phorbol ester aporeceptor in mouse brain cytosol Proc Natl Acad Sci USA 80 4208–4212

Marston FAO (1986) The purification of eukaryotic polypeptides synthsized in *Escherichia coli* Biochem J 240 1–12

Niedel JE, Kuhn LJ, Vandenbark GR (1983) Phorboldiester receptor copurifies with protein kinase C Proc Natl Acad Sci USA 80 36–40

Nishizuka Y (1986) Studies and perspectives of protein kinase C Science 233 305–312

Nishizuka Y (1988) The molecular heterogeneity of protein kinase C and its implications for cellular regulation Nature 334 661–665

Ono Y, Fujii T, Igarashi K, Kuno T, Tanaka C, Kikkawa U, Nishizuka Y (1989) Phorbol ester binding to protein kinase C requires cysteine-rich zinc-finger-like sequence Proc Natl Acad Sci USA 86 4868–4871

Parker PJ, Coussens L, Totty N, Rhee L, Young S, Chen E, Waterfield MD, Francke U, Ullrich A (1986) The complete primary structure of protein kinase C—the major phorbol ester receptor Science 233 853–859

Parker PJ, Mitchell F, Stabel S, Marais R, Ullrich A, Goris J (1987) Phosphorylation of protein kinase C Adv Prot Phosphatases 4 363–373

Parker PJ, Stabel S, Waterfield MD (1984) Purification to homogeneity of the protein kinase C from bovine brain-identity with the phorbol ester receptor EMBO J 3 953–959

Patel G, Stabel S (1989) Expression of a functional protein kinase C-γ using a baculovirus vector Purification and characterization of a single protein kinase C iso-enzyme Cell Signal 1 227–240

Pears CJ, Stabel S, Cazaubon S, Parker PJ (1992) Studies on the phosphorylation of protein kinase C-α Biochem J 283 515–518

Regazzi R, Fabbro D, Costa S, Eppenberger U (1986) Effects of tumor promoters on growth and on cellular redistribution of phospholipid/Ca^{2+}-dependent protein kinase in human breast cancer cells Int J Cancer 37 731–737

Sambrook J, Fritsch EF, Maniatis T (1989) In Ford N, Nolan C, Ferguson M (eds) "A Laboratory Manual" Cold Spring Harbor, NY Cold Spring Harbor Laboratory Press, A 3

Studier FW, Rosenberg AH, Dunn JJ, Dubendorff JW (1990) Use of T7 RNA polymerase to direct the expression of cloned genes Methods Enzymol 185 60–89

Wartmann M, Jans D, Parker P, Nagamine Y, Hemmings B, Jaken S, Eppenberger U, Fabbro D (1991) Overexpression of the a-type protein kinase (PK) C in LLC-PK1 cells does not lead to a proportional increase in the induction of two 12-O-tetradecanoylphorbol-13-acetate-inducible genes Cell Regul 2 491–502

Young S, Parker PJ, Ullrich A, Stabel S (1987) Down-regulation of protein kinase C is due to an increased rate of degradation Biochem J 244 775–779