Recombinant protein kinase C-γ phorbol binding domain upon microinjection blocked insulin-induced maturation of *Xenopus leavis* oocytes

Tadeusz Pawelczyk*, Andrzej Matecki, Anita Dettlaff

Department of Clinical Biochemistry, Medical University of Gdansk, 80-211 Gdansk, Poland

Received 4 December 1997; revised version received 12 January 1998

Abstract The second cysteine-rich (Cys-2) domain of rat brain PKC-γ regulatory region C1 (92–173) was expressed in *Escherichia coli* cells and purified. NMR studies of Cys-2 protein identified the phorbol and other phospholipid binding sites within this molecule (Xu, R.X., Pawelczyk, T., Xia, T-H. and Brown, S.T. (1997) Biochemistry 37, 10709–10717). Here, we tested the ability of this domain to bind other proteins. Using an overlay assay we show that the Cys-2 domain binds other proteins in *Xenopus* oocyte soluble fraction. Unlike the kinase activity, binding of Cys-2 to other proteins was detected in the absence of added phospholipids. Microinjection of Cys-2 protein into *Xenopus leavis* oocytes inhibited insulin-induced but not progesterone-induced maturation. the smallest dose that enhanced insulin-induced maturation was 0.45×10^{-12} mol injected Cys-2. These results demonstrate that the PKC-γ Cys-2 domain beside being the binding site for phorbol ester/DAG and phosphatidylserine binds also other proteins. The proteins that interact with Cys-2 domain of PKC are essential for insulin-induced maturation program in oocytes.

Key words: Protein kinase C; Cys-2 domain; Insulin; Maturation; Oocyte; *Xenopus leavis*

1. Introduction

Protein kinase C (PKC) is a family of at least 11 serine/threonine kinases that represent the key components of intracellular signal transduction [1,2]. Based on their structure and cofactor regulation the PKC isozymes have been classified into three groups, i.e. conventional (α, βI, βII, γ), novel (δ, ε, η, θ, μ) and atypical (ζ, λ). Members of the PKC family are a single polypeptide composed of an N-terminal regulatory region and a C-terminal catalytic region. Multiple, functionally distinct segments are present within the regulatory and catalytic segments. The regulatory region of conventional PKCs which participate in autoinhibition and transient activation of the enzyme have a C1, C2 and V1 domain [3]. The C1 domain contains a duplicated cys-rich motif (Cys-1, Cys-2) responsive to DAG/phorbol ester [4,5]. The C2 domain binds Ca^{2+} and acidic lipids [6]. However, more detailed studies of phorbol ester binding to individual segments of PKC-γ C1 region namely Cys-1 and Cys-2 revealed a complex mechanism involving phospholipids, phorbol esters and divalent cations [7,8]. These studies established that a single cysteine-rich domain (Cys-2) of PKC-γ contains regions necessary and sufficient for PS and calcium-dependent stereospecific interactions with phorbol esters [8,9]. Moreover, the Cys-2 domain appears to be sufficient to confer translocation of a fusion protein to lipid membrane in a phorbol ester and DAG-dependent fashion [8].

Upon activation of several signalling events in the cell, the PKC isozymes translocate to new cellular compartments [10–12]. With the use of immunofluorescence technic unique subcellular localization for individual PKCs has been demonstrated [13–15]. It has been postulated that in addition to being effector molecules, PKC substrates also bind to the kinase directly. Recently, several proteins associating with PKC have been discovered [16–18]. It was proposed that the regulatory domains of the PKCs contain important determinants of the PKC interactions with other proteins [19]. Such assumption is supported by the observation that binding of RACK1 to PKC-β takes place at the C2 region [15,16]. The calcium-dependent and phospholipid-independent binding of neuronal substrate GAP-43 to V0/C2 region of PKC-δ was also reported [20]. The ENH protein, which contains the cys-rich domains (LIM domain) binds to the PKC-β1 and PKC-ε through the interaction with the V1 region [21]. It was demonstrated that the PH domains of kinases Btk and Emt (members of Tec family protein tyrosine kinases) interact with PKC [22,23]. Recently, direct protein-protein interaction between the C1 region of PKC and PH domain of kinase Btk have been reported [24]. Moreover, interaction of these proteins was shown to be dependent on phorbol ester and phosphatidylinositol 4,5-bisphosphate binding. Our studies showed that the Cys-2 domain of PKC-γ binds some proteins in phospholipid-independent fashion, and the other proteins in phosphatidylserine-dependent manner (Pawelczyk, T., Kowara, R., Dettlaff, A. and Matecki, A., manuscript in preparation). We assumed that the Cys-2 peptide when injected into the cell should inhibit (by the competition) PKC-dependent signalling in this cell if these interactions are critical for that process. In order to examine our hypothesis we injected purified recombinant PKC-γ Cys-2 protein into *Xenopus leavis* oocytes. Presented data showed that injection of Cys-2 protein into oocytes at micromolar concentration blocked insulin-induced maturation.

2. Materials and methods

Insulin, 3-aminobenzoic acid ethyl ester methane sulfonate salt (Tricane), glutathione-agarose, and glutathione were from Sigma-Aldrich Sp. z o.o. (Poznan, Poland). Bovine plasminogen free thrombin was from Calbiochem-Novabiochem (La Jolla, USA). Collagenase type I was purchased from Boehringer (Mannheim, Germany).

*Corresponding author. Fax: +58 (344) 9653.
E-mail: tkpaw@amedec.amg.gda.pl

Abbreviations: DAG, diacylglycerol; GST, glutathione *S*-transferase; GVBD, germinal vesicle breakdown

PII S0014-5793(98)00054-4

The Cys-2 domain (92–173) was cloned into pGEM-2TKG vector and expressed in *E. coli* BL21(DE3) cells as a fusion protein as described previously [9]. The GST Cys-2 protein was purified on glutathione-agarose column, and the Cys-2 protein was cleaved from glutathione S-transferase (GST) by thrombin digestion. Cleaved Cys-2 protein was finally purified on Mono-S (HR 5/5) column as described [9].

Protein was determined by the method of Bradford [25] using bovine serum albumin as standard.

2.1. Preparation of tissue extracts

X. leavis oocytes (average diameter of 1.2 mm) were homogenized in two volumes of 10 mM HEPES-NaOH, pH 7.2, 5 mM $MgCl_2$, 25 mM KCl, 1 mM Pefabloc SC, 5 μM leupeptin, 1 μM aprotinin using a power-driven pestle. The homogenate was centrifuged at $120\,000\times g$ for 1 h. The resulting supernatant (in 1 ml portions) was stored at −20°C as the soluble fraction. The sediment from ultracentrifugation was suspended in 2 volumes of homogenization buffer supplemented with 200 mM NaCl and homogenized in a glass homogenizer with a motor-driven Teflon pestle. The resulting homogenate was supplemented with sodium chelate to a final concentration of 1.2%. After stirring for 60 min at 4°C, insoluble material was removed by centrifugation at $40\,000\times g$ for 1 h. The supernatant was stored (in 1 ml portions) at −20°C as the particulate fraction.

2.2. Antibodies

Polyclonal antibody to the PKC-γ Cys-2 protein was generated in rabbits. Rabbits were subcutaneously injected in the back of the neck with 400 μg of purified antigen in Freund's adjuvant followed by three boosts with 200 μg of antigen every 3 weeks. The antibody was purified by ammonium sulfate precipitation and by chromatography on protein A-agarose column.

2.3. Immunoblots

The samples (100 μg of protein) were separated on 10% SDS-PAGE according to Laemmli [26] and then electrophoretically transferred to nitrocellulose membrane. The membrane was blocked with 5% non-fat milk powder in phosphate-buffered saline (PBS) with 0.02% NaN_3 and then washed with PBS.

2.4. Overlay assay

The overlay assay was used as described previously by Susan Jaken's group [15] with minor modifications. The blocked membrane strips were incubated for 1 h in TBS (50 mM Tris-HCl, pH 7.4, 0.5 M NaCl) containing 100 μg/ml purified PKC-γ Cys-2 protein, 10 mg/ml bovine serum albumin, 1 mM Pefabloc SC, 5 μM leupeptin, 1 μM aprotinin, 1 mM EGTA, 1.1 mM $CaCl_2$. After incubation with Cys-2 protein, samples were washed with PBS containing required cofactors and fixed with 0.5% formaldehyde in PBS for 20 min. Next, the reactive aldehyde groups were blocked by incubation with 2% glycine in PBS for 20 min. The membrane strips were washed three times in TBS and incubated with rabbit anti-rat PKC-γ Cys-2 polyclonal antibodies. Immunostaining was done using alkaline phosphatase-conjugated goat anti-rabbit IgG, the chromogenic substrate 5-bromo-4-chloro-3-indoyl phosphate and Nitro Blue Tetrazolium.

2.5. Oocyte preparation

Oocytes were prepared following standard procedures [27]. Briefly, *X. leavis* (Blades Biological, England) were anesthetized by placing individual frogs in 0.1% Tricane for 30–60 min. Ovaries were removed from the frog, cut into small pieces and placed in Barth Medium (BM) without Ca^{2+} (110 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 2 mM $NaHCO_3$, 10 mM HEPES, pH 7.8). Oocytes were isolated by treating these pieces with type I collagenase (2 mg/ml). After extensive washing, healthy stage VI oocytes (approx. 1.2 mm diameter) were selected for assays. Either Cys-2 protein or buffer alone were injected (40 nl) into oocytes. Oocytes were cultured at 18°C in BM with 1 mM $CaCl_2$. Oocyte maturation was induced by the addition of 1 μM insulin [28] or 1 μg/ml progesterone [29]. Oocyte maturation was assessed at different times (in 1 h intervals) by the appearance of a white spot in the animal pole due to germinal vesicle (nuclear) breakdown (GVBD). In some cases, to confirm visual observations, nuclear breakdown was confirmed by dissection of trichloroacetic acid (10%) fixed oocytes [30].

3. Results

The process of oocyte maturation is initiated by the action of agonist at the oocyte surface. This is followed by activation of a cytoplasmic maturation-promoting factor which induces the observed events associated with maturation [31]. Depending on maturation inducer, different signalling events occurred in oocytes. The cAMP and Ca^{2+}-dependent signalling system is the most extensively studied system in the oocytes [32]. Progesterone, which is the physiological inducer of oocyte maturation triggers this signalling pathway. The key components of the progesterone-induced signalling are protein kinase A (PKA), the proto-oncogene product Mos and cyclin B [32]. On the other hand it has been demonstrated that oncoprotein Ras and protein kinase C (PKC) are important components of the insulin-induced maturation [33–36]. Peptides derived from the fragments of proteins that bind to the PKC-β (RACK1 and annexin I) affected the PKC translocation and insulin-induced maturation upon injection into *X. leavis* oocytes [15]. Our studies on Cys-2 domain (91–172) of PKC-γ revealed that this domain beside binding DAG/phorbol esters and phospholipids binds also other proteins in rat brain extracts (Pawelczyk, T., Kowara, R., Dettlaff, A. and Matecki, A., manuscript in preparation). Using an overlay assay system we were able to detect also in *X. leavis* oocyte soluble fraction proteins that bind to the Cys-2 protein (Fig. 1). However, no binding proteins were detected in the particulate fraction of oocytes. In order to evaluate if the protein interactions within Cys-2 domain of PKC are essential for insulin-induced GVBD we injected the purified recombinant Cys-2 protein into *X. leavis* oocytes. Microinjection of the Cys-2 protein inhibited insulin-induced GVBD (Fig. 2). This inhibition was dose dependent and after 23 h, only 30% of the oocytes reached maturation when microinjected with 3.6×10^{-12} mol of Cys-2 protein, as compared with maximal maturation at 11 h in control injected (10 mM HEPES, pH 7.5) oocytes (Fig. 2). The smallest dose that enhanced insulin-induced oocyte maturation was 0.45×10^{-12} mol of Cys-2. The average size of our oocytes was 1.2 mm. Thus, it can be calculated that the average oocyte volume was 0.9 μl. One can calculate that the Cys-2 protein affected the insu-

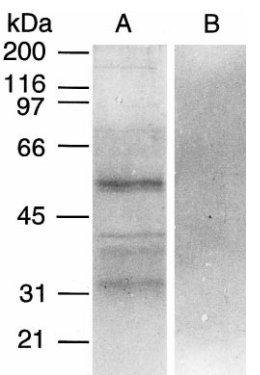

Fig. 1. Binding of Cys-2 protein to proteins in *Xenopus leavis* oocyte extract. The proteins (100 μg) from oocyte soluble (strip A) and particulate (strip B) fraction were separated by SDS-PAGE, transferred to nitrocellulose membrane and incubated with Cys-2 protein in the presence of 0.1 mM $CaCl_2$ as described in Section 2. Bound Cys-2 protein was detected with purified anti-Cys-2 antibody. The positions of molecular mass markers (in kDa) are indicated on the left.

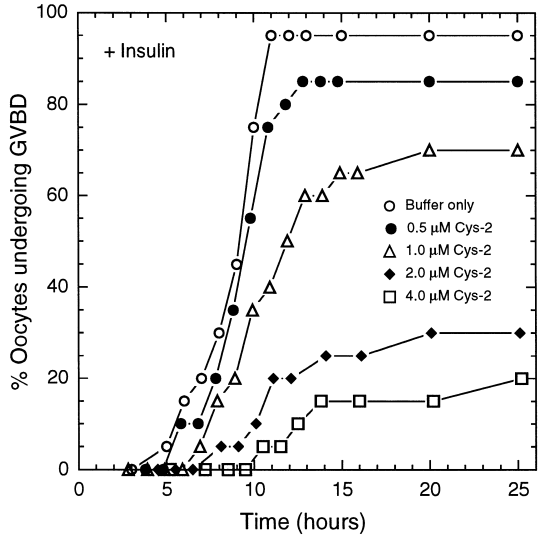

Fig. 2. Time course of insulin-induced *Xenopus leavis* oocyte maturation after injection of Cys-2 protein. Oocytes (in group of 20 for each experimental condition) were injected (40 nl) with: 10 mM HEPES, pH 7.5 (vehicle, ○), 0.45 pmol (●), 0.9 pmol (△), 1.8 pmol (◆), or 3.6 pmol (□) of Cys-2 protein. Insulin (1 μM) was added 1 h later, and the development of GVBD in time was monitored. Results are expressed as a percentage of oocytes reaching GVBD. The concentrations shown were calculated based on an average oocyte volume of 0.9 μl. Presented results are representatives of those obtained from four independent experiments.

lin-induced oocyte maturation at concentration as low as 0.5 μM. When the Cys-2 protein was injected to reach the concentration of 1 μM only 50% of the oocytes reached maturation at 11 h, as compared with maximal maturation (100%) observed at this time point in control injected oocytes (Fig. 2). We did not detect any changes in progesterone-induced oocyte maturation upon injection of Cys-2 protein (Fig. 3). In the presence of 1 μg/ml progesterone 100% oocytes reached maturation within 7 h regardless Cys-2 injection. Under employed conditions Cys-2 in doses up to 9×10^{-12} mol did not enhance progesterone-induced maturation of oocytes. These results demonstrate that the Cys-2 protein acts on specific critical step(s) in the oocyte maturation program induced by insulin but not by progesterone.

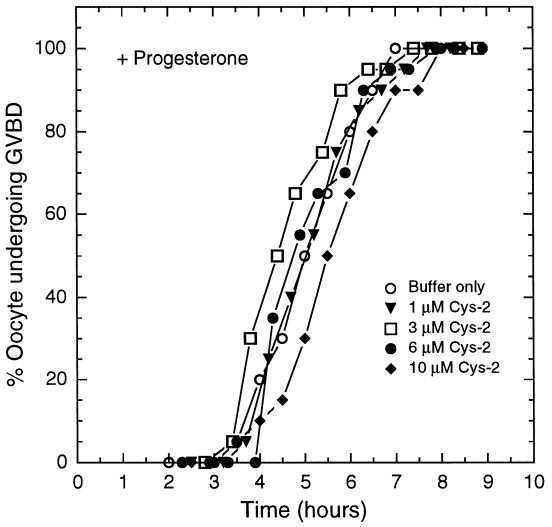

Fig. 3. Time course of progesterone-induced *Xenopus leavis* oocyte maturation after injection of Cys-2 protein. Conditions were as described in the legend to Fig. 2, except that oocytes were injected with: 10 mM HEPES, pH 7.5 (vehicle, ○), 0.9 pmol (▼), 2.7 pmol (□), 5.4 pmol (●), or 9 pmol (◆) of Cys-2 protein. Progesterone (1 μg/ml) was added 1 h later, and the development of GVBD in time was monitored. Essentially identical results were obtained in three other experiments.

4. Discussion

A number of recent studies suggest that PKC isozymes act on specific substrates by means of binding to variety of anchoring proteins as well as to other proteins that translocate the enzyme to certain regions of the cell [16,17]. Most of the identified to date proteins that bind PKC isozymes interact with them within the regulatory region of the kinase. The interaction within specific domains is the means by which each PKC isoform recognizes its specific substrate or binding protein. Such assumption is supported by several observations. Binding of RACK1 (receptor for activated C-kinase) to PKC-β takes place at the C2 region [36]. The peptide βC2-4 derived from the sequences of the interacting domains inhibits the PKC binding to RACK in vitro and inhibits the PKC-mediated function in vivo [15,37,38]. Introduction of εV1-2 octapeptide derived from the RACK binding site of PKC-ε into rat islets abolished glucose-induced translocation of PKC-ε [39]. These peptides were used as novel PKC isozyme-specific inhibitors to determine the specific function of individual isozymes [38,39]. The calcium-dependent and phospholipid-independent binding of neuronal substrate GAP-43 to PKC-δ takes place within V0/C2 region [20]. The protein ENH, which contains the cys-rich domains (LIM domains) binds to the PKC-β1 and PKC-ε through the interaction with the V1 region of PKC. This protein does not bind to PKC-α, δ and ζ [21]. Our results for the first time demonstrate that PKC-γ Cys-2 domain beside being the binding site for phorbol ester/DAG and phosphatidylserine binds also other proteins. The proteins that interact with Cys-2 domain are crucial components of insulin-induced but not progesterone-induced maturation signalling cascades in oocyte. We assume that injected Cys-2 protein binds to proteins in oocyte and inhibits by competition endogenous PKC. However, it should be emphasized that competition of Cys-2 protein with endogenous PKC for its physiological activator, i.e. diacylglycerol may also leads to inhibition of the processes dependent on PKC action. Additional studies to determine if the localization of the endogenous PKC is altered after Cys-2 injection are required to fully address this problem. Future studies should also lead to identification of the protein(s) that interact with Cys-2 domain, as well as to identification of a segment within Cys-2 domain that is responsible for the interaction with other proteins.

References

[1] Nishizuka, Y. (1992) Science 258, 607–614.
[2] Dekker, L.V. and Parker, P.J. (1994) Trends Biochem. Sci. 19, 73–77.
[3] Newton, A.C. (1995) J. Biol. Chem. 270, 28495–28498.
[4] Ohno, Y., Fujii, T., Igarashi, K., Kuno, T., Tanaka, C., Kokkawa, U. and Nishizuka, Y. (1989) Proc. Natl. Acad. Sci. USA 86, 4868–4871.
[5] Quest, A.F.G., Bardes, E.S.G. and Bell, R.M. (1994) J. Biol. Chem. 269, 2961–2970.

[6] Newton, A.C. (1995) Curr. Biol. 5, 973–976.
[7] Quest, A.F., Bardes, E.S.G. and Bell, R.M. (1994) J. Biol. Chem. 269, 2953–2960.
[8] Quest, A.F.G. and Bell, R.M. (1994) J. Biol. Chem. 269, 20000–20012.
[9] Xu, R.X., Pawelczyk, T., Xia, T-H. and Brown, S.C. (1997) Biochemistry 36, 10709–10717.
[10] Shoji, M., Girard, P.R., Mazzei, G.J., Vogler, W.R. and Kuo, J.F. (1986) Biochem. Biophys. Res. Commun. 135, 1144–1149.
[11] Misra, U.K. and Sahyoun, N. (1987) Biochem. Biophys. Res. Commun. 145, 760–768.
[12] Kiley, S.C. and Jaken, S. (1990) Mol. Endocrinol. 4, 59–68.
[13] Lehel, C., Olah, Z., Jakab, G. and Anderson, W.B. (1995) Proc. Natl. Acad. Sci. USA 92, 1406–1410.
[14] Kiley, S.C. and Parker, P.J. (1995) Cell Sci. 108, 1003–1016.
[15] Ron, D. and Mochly-Rosen, D. (1994) J. Biol. Chem. 269, 21395–21398.
[16] Mochly-Rosen, D. (1995) Science 268, 247–251.
[17] Jaken, S. (1996) Curr. Opin. Cell. Biol. 8, 168–173.
[18] Faux, M.C. and Scott, J.D. (1996) Cell 85, 9–12.
[19] Liao, L., Hyatt, S.L., Chapline, C. and Jaken, S. (1994) Biochemistry 33, 1229–1233.
[20] Dekker, L.V. and Parker, P.J. (1997) J. Biol. Chem. 272, 12747–12753.
[21] Kuroda, S., Tokunaga, C., Kiyohara, Y., Chiguchi, O., Konishi, H., Mizuno, K., Gill, G.N. and Kikkawa, U. (1996) J. Biol. Chem. 271, 31029–31032.
[22] Yao, L., Kawakami, Y. and Kawakami, T. (1994) Proc. Natl. Acad. Sci. USA 19, 9175–9179.
[23] Kawakami, Y., Yao, L., Tashiro, M., Gibson, S., Mills, G.B. and Kawakami, T. (1995) J. Immunol. 155, 3556–3562.
[24] Yao, L., Suzuki, H., Ozawa, K., Deng, J., Lehel, C., Fukamachi, H., Anderson, W.B., Kawakami, Y. and Kawakami, T. (1997) J. Biol. Chem. 272, 13033–13039.
[25] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[26] Leammli, U.K. (1970) Nature 227, 680–685.
[27] Colman, A. (1984) in: Transcription and Translation: A Practical Approach (Hames, B.D. and Higgins, S.J., Eds.), pp. 271–300, URL Press, Washington, DC.
[28] Maller, J.L. and Koontz, H. (1981) Dev. Biol. 85, 309–316.
[29] Baulieu, E-E., Godeau, F., Schorderet, M. and Schorderet-Slatkine, S. (1978) Nature (Lond.) 275, 593–598.
[30] Billah, M.M., Eckel, S., Mullmann, T.J., Egan, R.W. and Siegel, M.I. (1997) J. Biol. Chem. 264, 17069–17077.
[31] Smith, D. (1989) Development 107, 685–699.
[32] Matten, W.T. and Vande Woude, G.F. (1994) Dev. Biol. 5, 173–181.
[33] Korn, L.J., Siebel, C.W., McCormic, F. and Roth, R.A. (1987) Science 236, 840–843.
[34] Deshpande, A. and Kung, H.F. (1987) Mol. Cell. Biol. 7, 1285–1288.
[35] Domingues, I., Diaz-Meco, M.T., Municio, M.M., Berra, E., Garcia de Herreros, A., Cornet, M.E., Santz, L. and Moscat, J. (1992) Mol. Cell. Biol. 12, 3776–3783.
[36] Ron, D., Chen, C.H., Caldwell, J., Jamieson, L. and Mochly-Rosen, D. (1994) Proc. Natl. Acad. Sci. USA 91, 839–843.
[37] Ron, D., Luo, J. and Mochly-Rosen, D. (1995) J. Biol. Chem. 270, 24180–24187.
[38] Yedovitzky, M., Mochly-Rosen, D., Johnson, J.A., Gray, O.M., Ron, D. and Nesher, R. (1997) J. Biol. Chem. 272, 1417–1420.
[39] Johnson, J.A., Gray, M.O., Chen, C.H. and Mochly-Rosen, D. (1996) J. Biol. Chem. 271, 24962–24966.