# High-efficiency expression of rat protein kinase C-γ in baculovirus-infected insect cells

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We have expressed rat protein kinase C-γ in insect cells using a baculovirus vector. The yield of expressed protein kinase C-γ is about 4% of total protein. The recombinant protein shows a prominent band at about 80 kDa on SDS-polyacrylamide gels, which can be identified as protein kinase C-γ by Western blotting using monoclonal antibodies against protein kinase C-γ. Upon incubation with [γ-$^{32}$P]ATP and in the presence of $Ca^{2+}$, phosphatidylserine and diacylglycerol this protein autophosphorylates. Its enzyme activity shows the characteristic properties of mammalian protein kinase C.

Protein kinase C; Baculovirus; Gene expression

## 1. INTRODUCTION

Protein kinase C (PKC), originally defined as a $Ca^{2+}$-, PS- and $diC_{18:1}$-dependent serine and threonine kinase, is a ubiquitously distributed enzyme. Several cloned subspecies of PKC-cDNAs can be divided into two groups [1]. Isoforms belonging to the first group, PKC-α, -βI, -βII and -γ, are activated by $Ca^{2+}$, PS and $diC_{18:1}$ [2–6], while the recently cloned and expressed isoforms, PKC-δ, -ε, -ε′ and -ζ, which are likely to be $Ca^{2+}$-independent form the second group [7–11].

The expression pattern of the PKC subspecies has been determined in several tissues and some cell lines [12–17]. So far, expression of the PKC-γ isoform has been detected only in the nervous tissue [13,18].

A detailed characterization and elucidation of the specific role of PKC subspecies has not been possible because of the difficulty in obtaining highly purified PKC isozymes. In order to get sufficient amounts of one specific PKC isozyme without cross-contamination by other mammalian PKC subspecies, we have expressed rat PKC-γ in the baculovirus-insect-cell-system [19,20], as has been reported recently for several other cDNAs of PKC isozymes [11,21,22].

Here we report on high-yield expression of rat PKC-γ in insect cells infected with a recombinant baculovirus containing the rat PKC-γ cDNA [23]. The amount of active, recombinant PKC-γ in crude cell extracts was about 4% of the total protein. The characteristic properties of this enzyme are described.

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*Abbreviations:* PKC, protein kinase C; EGTA, [ethylenebis-(oxyethylenenitrilo)]tetraacetic acid; PMA, phorbol 12-myristate 13-acetate; PS, phosphatidylserine; $diC_{18:1}$, *sn*-1,2-dioleoyl-glycerol; PMSF, phenylethylsulfonyl fluoride; PBS, phosphate-buffered saline

## 2. MATERIALS AND METHODS

### 2.1. *Cells and viruses*

*Spodoptera frugiperda* cells (Sf158) and wild-type *Autographa californica* nuclear polyhedrosis virus (AcMNPV) were kindly provided by K. Herrenknecht and Dr J. Kupsch (MPI, Freiburg, FRG). Cells were grown at 27°C in TC-100 medium (Gibco) containing 10% fetal bovine serum (Gibco). After viral infection 2.5 mg/l fungizone (Gibco) and 50 mg/l gentamycin (Gibco) were added. Procedures for cell culture, viral infection and isolation of viral genomic DNA have been described in detail by Summers and Smith [24].

### 2.2. *Plasmid constructs*

Dr M. Summers (Texas A&M University, College Station, TX, USA) generously provided the baculovirus expression vector pVL1393. The transfer vector pVL1393-PKC-γ was constructed as follows. The 2.6 kilobase pair PKC-γ *Eco*RI fragment was isolated from pGem-3 (kindly provided by Dr J. Knopf, Genetics Institute, Cambridge, MA, USA) and ligated into pVL1393 cut with *Eco*RI. The orientation of the PKC-γ insert was determined by restriction analysis. Standard procedures were carried out as described [24,25].

### 2.3. *Isolation of recombinant baculovirus*

Recombinant baculovirus was produced by cotransfecting Sf158 cells with purified genomic AcMNPV-DNA and pVL1393-PKC-γ expression vector [24]. Recombinant polyhedra negative virus, containing the PKC-γ insert, was isolated by visual screening of plaques [24] under a phase contrast microscope (Leitz Labovert FS). After three rounds of plaque purification, recombinant plaques were hybridized with the $^{32}$P-labeled 2.6 kilobase pair PKC-γ cDNA fragment. All polyhedra negative plaques gave a strong signal in the autoradiograph [24].

### 2.4. *Cell lysis*

Cells infected with recombinant AcMNPV-PKC-γ virus were

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harvested 72–90 h post-infection and pelleted at 1000 × *g* for 10 min. After washing two times in PBS the cells were resuspended in lysis buffer (20 mM Tris-HCl, pH 7.5, 10 mM EGTA, 2 mM EDTA, 3 mM DTT, 50 μg/ml PMSF) and disrupted on ice with 20 strokes in a Potter homogenizer [21].

2.5. *Immunoblotting*

Proteins were separated on SDS-polyacrylamide (8%) gels [25] and then transfered to nitrocellulose paper (Schleicher & Schuell) [26]. After blocking residual protein binding sites with bovine serum albumin, the cellulosenitrate blot was probed with anti rabbit brain PKC-γ monoclonal antibody MC-1a (Seikagaku Kogyo Co., Tokyo, Japan) overnight at 4°C. Incubation with second antibody was performed with anti-mouse alkaline phosphatase conjugate (Promega) for 45 min at room temperature [27].

2.6. *PKC activity assay*

PKC-γ activity was measured according to Schächtele et al. [28] in 200 μl incubation medium, consisting of 50 mM Hepes-NaOH, pH 7.5, 1 mM EDTA, 1.25 mM EGTA, 1.32 mM $CaCl_2$ (corresponds to 4 μM free $Ca^{2+}$), 5 mM $MgCl_2$, 0.2 mg/ml histone III-S (Sigma), 10 μM ATP, 1 μCi/ml [γ-$^{32}$P]ATP (5000 Ci/mmol; Amersham) and 0.2–0.3 μg protein of crude cell extract containing recombinant PKC-γ (diluted in 10 mM Hepes-NaOH, pH 7.5, 1 mM DTT). Activators and inhibitors were added as indicated in Table I. Experiments without $Ca^{2+}$ were carried out by adding 2.25 mM EGTA. After 5 min of incubation at 30°C reactions were stopped with 2 ml of 10% ice-cold trichloroacetic acid and filtered through nitrocellulose filters (Sartorius). $^{32}$P-Incorporation was determined by Cerenkov counting. One unit of PKC activity is defined as the amount of enzyme catalyzing the incorporation of 1 nmol of phosphate from ATP into histone III-S under standard assay conditions.

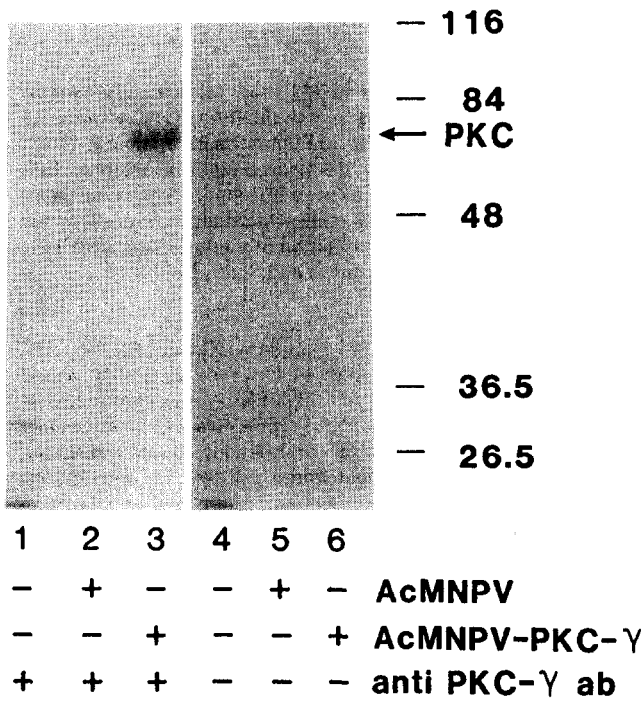


Fig. 1. Western blot analysis of rat PKC-γ from AcMNPV-PKC-γ-infected insect cells. The insect cells were either uninfected (lanes 1 and 4), or infected with wild-type baculovirus (lanes 2 and 5), or infected with AcMNPV-PKC-γ (lanes 3 and 6). Only lanes 1–3 were incubated with anti PKC-γ antibody (anti PKC-γ ab). 20 μg protein of cell lysate was loaded on each lane. Relative molecular masses in kDa of marker proteins and position of PKC are indicated at the right.

2.7. *Autophosphorylation*

The reaction mixture for the autophosphorylation was the same as described above but containing 5 μg protein of cell lysate and no histone. For stimulation of PKC activity, 1 μg PS (Sigma) and 0.2 μg $diC_{18:1}$ (Sigma) were added. Reactions were carried out at 30°C for 50 min [29]. Proteins were precipitated with 100 μl of ice-cold 100% trichloroacetic acid and pelleted for 10 min (4°C) at 18 000 × *g*. After washing twice with acetone, the pellet was resuspended in 50 μl 4 × SDS sample buffer modified from Laemmli [25]. After electrophoresis on SDS-polyacrylamide (8%) gels, proteins were silver-stained according to Heukeshoven and Dernick [30], the gel was dried and PKC autophosphorylation was identified by autoradiography.

## 3. RESULTS AND DISCUSSION

Infection of Sf 158 cells with the recombinant baculovirus (AcMNPV-PKC-γ), harboring the cDNA of rat PKC-γ, resulted in high levels of PKC-γ expression. Using monoclonal antibodies against PKC-γ for immunoblotting, a signal at about 80 kDa could only be detected in extracts from insect cells infected with AcMNPV-PKC-γ (Fig. 1, lane 3). Uninfected insect cells (Fig. 1, lane 1) and wild-type baculovirus-infected insect cells (Fig. 1, lane 2) gave no signal at 80 kDa. Control incubation, using only the second antibody, did not result in a specific signal at 80 kDa (Fig. 1, lanes 4–6). Faint signals occurring in the lower molecular mass range (below 48 kDa) with extracts from uninfected cells (Fig. 1, lane 1) were due to non-specific binding of the second antibody since the signals also occurred without incubation with the first antibody (Fig. 1, lane 4).

On a silver-stained SDS-polyacrylamide gel, a protein band at about 80 kDa was visible only in AcMNPV-

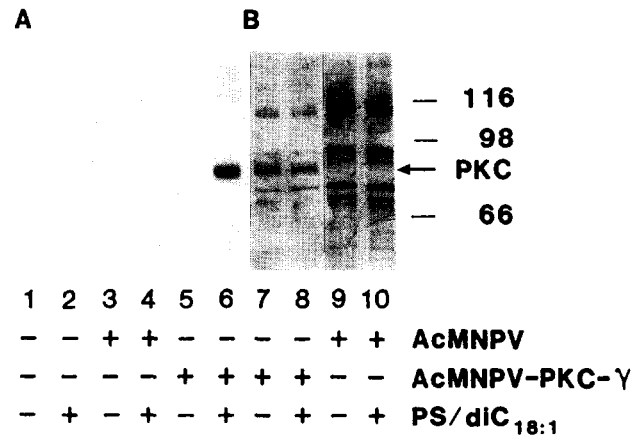


Fig. 2. Silver-stained SDS-polyacrylamide gel electrophoresis and autophosphorylation of rat PKC-γ expressed in AcMNPV-PKC-γ-infected insect cells. The insect cells were either uninfected (lanes 1 and 2), or infected with wild-type baculovirus (lanes 3, 4, 9 and 10), or infected with AcMNPV-PKC-γ (lanes 5, 6, 7 and 8). Autophosphorylation (A) was done in the presence of PS and $diC_{18:1}$ (lanes 2, 4 and 6) and absence of PS and $diC_{18:1}$ (lanes 1, 3 and 5). The silver-stained gel (B) contained 5 μg of protein per lane. This same gel was autoradiographed as shown in (A). Relative molecular masses in kDa of marker proteins and position of PKC are indicated at the right.

PKC-$\gamma$-infected cells (Fig. 2B, lanes 7 and 8) but not in wild-type baculovirus-infected cells (Fig. 2B, lanes 9 and 10). The PKC-$\gamma$ only sometimes resolved as a doublet on SDS-polyacrylamide gels as described by Patel and Stabel [21].

Autophosphorylation of recombinant PKC-$\gamma$ from AcMNPV-PKC-$\gamma$-infected insect cells resulted in a strong signal at 80 kDa in the presence of PS and $diC_{18:1}$ (Fig. 2A, lane 6), only a weak signal could be detected without PS and $diC_{18:1}$ (Fig. 2A, lane 5). In uninfected or wild-type-infected cells no such signal could be seen either in the presence or absence of PS and $diC_{18:1}$ (Fig. 2A, lanes 1–4). The weak signals above 116 kDa (Fig. 2A, lanes 1–6) and below 66 kDa (Fig. 2A, lanes 1–4) were probably due to a PS and $diC_{18:1}$-independent protein kinase in the insect cells.

PKC activity could be detected in crude extracts from Sf 158 cells infected with AcMNPV-PKC-$\gamma$ (Table I). Without the activators, PS and $diC_{18:1}$, only a low and $Ca^{2+}$-independent activity could be detected. Addition of PS resulted in a pronounced $Ca^{2+}$-dependent increase whereas addition of $diC_{18:1}$ enhanced the activity in a more or less $Ca^{2+}$-independent manner. Addition of PS and $diC_{18:1}$ or PS and PMA resulted in a maximal, $Ca^{2+}$-dependent activation. It should be noted that the PKC-$\gamma$ activity in the presence of PS and $diC_{18:1}$ but in the absence of $Ca^{2+}$ is considerably elevated (Table I). To exclude contaminating $Ca^{2+}$ in the incubation medium, we have added EGTA up to a final concentration of 6.25 mM without effecting PKC-$\gamma$ activity (data not shown). A similar elevated activity in the absence of $Ca^{2+}$ was observed when PKC-$\gamma$ was stimulated by $diC_{18:1}$ or PS and PMA (Table I). These results require further investigation.

Staurosporine strongly inhibited both basal and PS and $diC_{18:1}$-enhanced activity. Incubation with trypsin for 5 min at 20°C fully activated the recombinant PKC-$\gamma$ even in the absence of any activator. Assuming a specific activity of 1500 units/mg of purified PKC-$\gamma$ [21] and the measured PKC activity of 65.7 units/mg protein (Table I), we calculated that about 4% of the total protein was expressed as PKC-$\gamma$. In extracts from uninfected or wild-type-infected cells no activation of protein phosphorylation in the presence of PS and $diC_{18:1}$ could be seen (data not shown).

These results demonstrate the utility of the baculovirus expression system for highly efficient production of functionally active rat PKC-$\gamma$. This system provides sufficient material to allow detailed biochemical and structural analysis.

Table I

Activity of recombinant rat PKC-$\gamma$ from AcMNPV-PKC-$\gamma$-infected insect cells

| Activators/inhibitors | PKC activities (units/mg) | |
|---|---|---|
| | With calcium | Without calcium |
| None | 6.5 | 5.7 |
| PS (5 $\mu$g/ml) | 45.3 | 8.8 |
| $diC_{18:1}$ (1 $\mu$g/ml) | 19.9 | 17.4 |
| PS (5 $\mu$g/ml), $diC_{18:1}$ (1 $\mu$g/ml) | 65.7 | 44.4 |
| PS (5 $\mu$g/ml), PMA (0.2 $\mu$M) | 52.3 | 34.4 |
| Staurosporine (0.1 $\mu$M) | 3.4 | 1.9 |
| PS (5 $\mu$g/ml), $diC_{18:1}$ (1 $\mu$g/ml), staurosporine (0.1 $\mu$M) | 10.4 | 7.4 |
| Trypsin (0.4 $\mu$g/ml) for 5 min at 20°C | 55.4 | 54.2 |

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