

PKC- θ *in vitro* Kinase Activity Assay

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[Abstract] Protein kinase C- θ (PKC- θ), a member of the Ca²⁺-independent PKC subfamily of kinases, serves as a regulator of T cell activation by mediating the T cell antigen receptor (TCR)- and coreceptor CD28-induced activation of the transcription factors NF- κ B and AP-1 and, to a lesser extent, NFAT, and, subsequently, interleukin 2 (IL-2) production and T cell proliferation. In T cells, TCR and CD28 stimulation-induced activation of PKC- θ is the integrated result of diacylglycerol-mediated membrane recruitment, GLK-mediated phosphorylation at activation loop, CD28, Lck, and sumoylation-mediated central immunological synapse localization (Wang *et al.*, 2015; Monks *et al.*, 1997; Kong *et al.*, 2011; Isakov and Altman, 2012; Chuang *et al.*, 2011). Phosphatidylserine (PtdSer) and the phorbol ester Phorbol 12-myristate 13-acetate (PMA, a surrogate of diacylglycerol [DAG]) are the cofactors for the Ca²⁺-independent PKC subfamily that bind to PKC directly and activate it by changing its conformation (Nishizuka, 1995). A protocol to analyze the PKC- θ kinase activity *in vitro* is described here. Myelin basic protein is used as the substrate and its phosphorylation is detected by the incorporation of radioactive phosphate into the substrate, which is analyzed by a laser scanner.

[Background] Specified by their divergent regulatory domains, the PKC family can be divided into distinct subgroups: the conventional PKCs (cPKCs, comprising PKC- α , β and γ), the novel PKCs (nPKCs, including PKC- δ , ϵ , θ and η) and the atypical PKCs (aPKCs, PKC ζ and ξ). The cPKCs are activated by a combination of diacylglycerol, phospholipid and Ca²⁺. The nPKCs are activated by diacylglycerol and phorbol esters but do not require Ca²⁺. In contrast, the aPKCs do not depend on Ca²⁺ or diacylglycerol for activation (Rosse *et al.*, 2010). Here we used anti-Myc or anti-PKC- θ to isolate PKC- θ from the cell lysates, then performed the radiolabeled ATP based kinase assay in the Reaction buffer containing PMA, PtdSer and EGTA (a selective chelator for Ca²⁺). The assay protocol described here is quick, sensitive and specific, provides a direct measurement of PKC- θ activity. This protocol could be modified to analyze the activity of other nPKC isoforms.

Materials and Reagents

1. Jurkat E6.1 cells or HEK293T cells
2. Phosphate-buffered saline (PBS) (Thermo Fisher Scientific, Gibco™, catalog number: 10010023)
3. RPMI-1640 medium (GE Healthcare, HyClone™, catalog number: SH30027.01)

4. Heat-inactivated fetal bovine serum (FBS) (Thermo Fisher Scientific, Gibco™, catalog number: 10270-106)
5. Anti-PKC- θ (Santa Cruz Biotechnology, catalog number: sc-1875)
6. Anti-Myc (9E10) (Santa Cruz Biotechnology, catalog number: sc-40)
7. Protein G sepharose (GE Healthcare, catalog number: 17-0618-01)
8. Phosphatidylserine from PepTag® Non-Radioactive PKC assay kit (Promega, catalog number: V5330)
9. Tris (Sangon Biotech, catalog number: A600194)
10. Sodium chloride (NaCl) (Sigma-Aldrich, catalog number: S3014)
11. Ethylenediaminetetraacetate (EDTA) (Sangon Biotech, catalog number: A610185)
12. Nonidet P40 (Sangon Biotech, catalog number: A600385)
13. Aprotinin (EMD Millipore, catalog number: 616370)
14. Leupeptin (Calbiochem, catalog number: 108976)
15. Phenylmethanesulfonyl fluoride (PMSF) (Sigma-Aldrich, catalog number: P7626)
16. Sodium pyrophosphate tetrabasic decahydrate (Na₂P₂O₇) (Sigma-Aldrich, catalog number: S6422)
17. Sodium orthovanadate (Na₃VO₄) (Sigma-Aldrich, catalog number: 567540)
18. HEPES (Thermo Fisher Scientific, Gibco™, catalog number: 11344041)
19. Magnesium chloride (MgCl₂) (Sigma-Aldrich, catalog number: M4880)
20. EGTA (Sigma-Aldrich, catalog number: E3889)
21. ATP (Sigma-Aldrich, catalog number: A2383)
22. [γ -³²P]ATP (PerkinElmer, catalog number: NEG002Z001MC)
23. Myelin basic protein (MBP) (Sigma-Aldrich, catalog number: M1891)
24. PMA (Sigma-Aldrich, catalog number: P1585)
25. Bromophenol blue (Bio-Rad, catalog number: 1610404)
26. DL-dithiothreitol (DTT) (Sigma-Aldrich, catalog number: D9779)
27. Sodium dodecyl sulfate (SDS) (Sigma-Aldrich, catalog number: L3771)
28. Glycerol (Sangon Biotech, catalog number: A100854)
29. ATP-competitive PKC- θ inhibitor rottlerin (EMD Millipore, catalog number: 557370)
30. DMSO
31. Lysis buffer (see Recipes)
32. PKC- θ kinase buffer (see Recipes)
33. Reaction buffer (see Recipes)
34. 5x SDS-PAGE loading buffer (see Recipes)

Equipment

1. Typhoon Trio⁺ system (GE Healthcare)
2. Humidified CO₂ incubator
3. Laminar air flow bio-safety cabinet

4. Centrifuge (Eppendorf, model: 5417R)
5. Phosphor-storage screen (GE Healthcare)
6. Sonicator (Sonics, model: VCX-130)
7. Thermomixer (Eppendorf)
8. Exposure cassettes (GE Healthcare)

Software

1. Typhoon Scanner Control software

Procedure

1. Harvest 5×10^6 Jurkat E6.1 cells or 1×10^6 HEK293T cells transfected with vector, wild type (WT), kinase-dead mutant (K409R) and sumoylation deficient mutant (2KR [K325R, K506R]) of Myc-PKC- θ (indicated in Figure 2) by centrifugation ($300 \times g$, 3 min, 25°C).
2. Wash the cells once with 1 ml ice cold PBS.
3. Centrifuge the cells at $300 \times g$ for 3 min at 25°C .
4. Add 300-500 μl lysis buffer to the cell pellets, vortex for 1-2 min.
5. Place the tube on ice for 10 min.
6. Centrifuge at $8,500 \times g$ for 10 min at 4°C .
7. Transfer the clear supernatant to a new microcentrifuge tube.
8. Immunoprecipitate endogenous or transfected PKC- θ by adding 2.5 μl anti-PKC- θ or anti-Myc antibodies into the clear supernatant obtained in step 7. Incubate overnight at 4°C with rotation. Then add 30 μl protein G sepharose beads (50% [v/v] in PBS) and incubate for 2-4 h at 4°C with rotation.
9. Centrifuge for 5 min at $8,500 \times g$ at 4°C , carefully remove the supernatant. Wash the immunoprecipitates extensively by adding 1 ml lysis buffer and vortexing for 3-5 min.
10. Repeat step 9.
11. Centrifuge for 5 min at $8,500 \times g$ at 4°C , carefully remove the supernatant. Wash the immunoprecipitates extensively by adding 1 ml PKC- θ kinase buffer and vortexing for 3-5 min.
12. Repeat step 11.
13. Centrifuge for 5 min at $8,500 \times g$ at 4°C , carefully remove the supernatant and resuspend the pellet in 25 μl of the reaction buffer.
14. Incubate samples in the Thermomixer with gentle shaking at 300 rpm for 30 min at 30°C . Stop the reaction by adding 6.25 μl 5x SDS-PAGE loading buffer.
15. Heat samples at 95°C for 10 min and subject the samples to 15% SDS-PAGE, then cover the gel with plastic wrap to minimize the chance of radioactive contamination.
16. Open the exposure cassette, place the wrapped gel on the internal surface of the cassette, and then place the phosphor-storage screen on top the wrapped gel with the phosphor (white) side

facing down onto the gel. Close and lock the cassette, place it for 30 min to 6 h at room temperature and then take the phosphor-storage screen out of the cassette avoiding direct light. Scan the screen with the Typhoon Trio⁺ system.

17. (Optional) A pilot experiment to verify the specificity of this kinase assay is recommended. In detail, HEK293T cells were transfected with wild type Myc-PKC- θ , which has proven to be an active kinase as T538, S676, and S695 are constitutively phosphorylated on recombinant PKC- θ isolated from HEK293T expression systems (Wang *et al.*, 2012). 24 h after transfection, Myc-PKC- θ was immunoprecipitated and subjected to the kinase assay as described previously. When doing the kinase assay, the ATP-competitive PKC- θ inhibitor or DMSO was added into the reaction buffer which served as the negative and the positive control individually.

Data analysis

1. The kinase assay system works specifically if there is strong signal for the DMSO added sample while no signal for the PKC- θ inhibitor added sample in the pilot experiment.
2. Analyze signals with Typhoon Scanner Control software as followed:
 - a. Double-click the Scanner Control shortcut icon on the desktop.
 - b. After the current instrument state is 'Ready', select the acquisition mode as 'Storage Phosphor' (Figure 1).

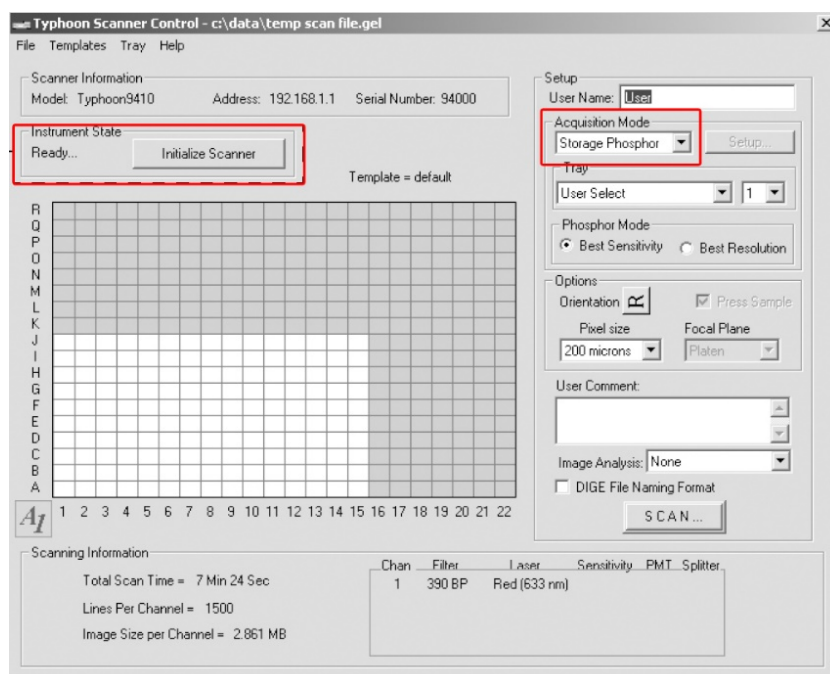


Figure 1. The scanner control window

- c. Place the storage phosphor screen in the Typhoon instrument. Remove the storage phosphor screen from the exposure cassette. Keep the screen face down. Position the

- screen on the glass platen so that the A1 corner of the screen aligns with the A1 corner of the glass platen. Close the sample lid.
- d. Select the grid area and pixel size, click 'SCAN' to start scanning.
 - e. Save the data and evaluate the results. A Representative data for the samples from transfected HEK293T cells was shown in Figure 2.

Representative data

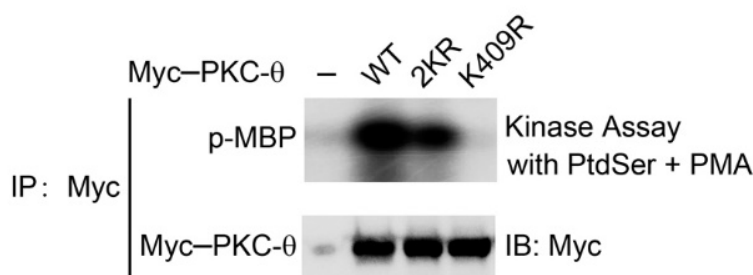


Figure 2. *In vitro* kinase assay of Myc-tagged PKC- θ . Myc-PKC- θ -WT, -2KR (K325R, K506R) or -K409R were immunoprecipitated (IP) from lysates of HEK293T cells transfected with empty vector or the indicated Myc-tagged PKC- θ plasmids, MBP was used as a substrate in the presence of PtdSer/PMA cofactors (upper panel). Aliquots of the IPs were immunoblotted with anti-Myc to confirm similar expression levels of PKC- θ (bottom panel).

Notes

Phosphatidylserine easily aggregates to form micelles. To achieve maximal PKC- θ activation, sonicate the phosphatidylserine solution with a probe sonicator for 20-30 sec or until warm to disrupt the micelles.

Recipes

1. Lysis buffer
 - 20 mM Tris-HCl (pH 7.5)
 - 150 mM NaCl
 - 5 mM EDTA
 - 1% Nonidet P40
 - 10 μ g/ml aprotinin
 - 10 μ g/ml leupeptin
 - 1 mM PMSF
 - 5 mM NaPPi
 - 1 mM Na_3VO_4

2. PKC- θ kinase buffer
 - 20 mM HEPES
 - 10 mM MgCl₂
 - 0.1 mM EGTA
3. Reaction buffer
 - 5 μ Ci [γ -³²P]ATP, 20 μ M ATP
 - 1 μ g of myelin basic protein (MBP)
 - 10 μ M PMA cofactors
 - 200 μ g/ml phosphatidylserine (PtdSer) (presence or absence)
4. 5x SDS-PAGE loading buffer
 - 0.25% bromophenol blue
 - 0.5 M DTT
 - 50% glycerol
 - 10% SDS
 - 0.25 M Tris-HCl (pH 6.8)

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