Caspase processing activates atypical protein kinase C ζ by relieving autoinhibition and destabilizes the protein

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Treatment of HeLa cells with tumour necrosis factor α (TNF α) induced caspase processing of ectopic PKC (protein kinase C) ζ , which converted most of the holoenzyme into the freed kinase domain and increased immune-complex kinase activity. The goal of the present study was to determine the basis for the increased kinase activity that is associated with caspase processing of PKC ζ . Atypical PKC ι is largely identical with PKC ζ , except for a 60-amino-acid segment that lacks the caspase-processing sites of the ζ isoform. Replacement of this segment of PKC ζ with the corresponding segment of PKC ι prevented caspase processing and activation of the kinase function. Processing of purified recombinant PKC ζ by caspase 3 *in vitro* markedly increased its kinase activity. Caspase processing activated PKC ζ *in vitro* or intracellularly without increasing the phosphorylation of Thr⁴¹⁰ of PKC ζ , which is required for catalytic competency.

The freed kinase domain of PKC ζ had a much shorter half-life than the holoenzyme in transfected HeLa cells and in non-transfected kidney epithelial cells. Treatment with TNF α shortened the half-life of the kinase domain protein, and proteasome blockade stabilized the protein. Studies of kinase-domain mutants indicate that a lack of negative charge at Thr⁴¹⁰ can shorten the half-life of the freed kinase domain. The present findings indicate that the freed kinase domain has substantially higher kinase activity and a much shorter half-life than the holoenzyme because of accelerated degradation by the ubiquitin–proteasome system.

Key words: apoptosis, atypical protein kinase C, caspase, lactacystin, proteasome, tumour necrosis factor (TNF).

INTRODUCTION

The PKC (protein kinase C) family of phospholipid-dependent serine/threonine kinases includes two isoforms, ζ and ι/λ , which are atypical because they have only half of the C1 domain and hence are not activated by C1 ligands, such as DAG (diacylglycerol), phorbol ester tumour promoters or bryostatins [1–4]. The ζ and ι/λ isoforms have a similar amino acid sequence, except for the last 60-residue segment of the N-terminal regulatory domain. Importantly, the 60-residue unique segment of PKC ζ includes the caspase-processing sites, which are absent from the ι isoform. Hence, following the induction of apoptosis, the ζ , but not the ι/λ isoform of PKC undergoes processing by caspases, which are a family of highly specific cysteine proteases that implement a cell-autonomous death pathway [5–8]. Processing of PKC ζ occurs chiefly, but not exclusively, at two aspartate residues (residues 210 and 239), which separates the regulatory and catalytic domains, and increases PKC ζ activity [6]. The biochemical basis for the activation of the PKC ζ kinase function that accompanies production of the freed kinase domain has not been elucidated.

Recently, we reported that the freed kinase domain is essentially devoid of kinase activity unless it is phosphorylated at Thr⁴¹⁰ by 3-phosphoinositide-dependent kinase-1 (PDK-1) [9]. Phosphorylation of Thr⁴¹⁰ of full-length PKC ζ by PDK-1 had previously been shown to be necessary and sufficient to activate the kinase function [10,11]. Although the only proven regulation of atypical PKCs is their requirement for phosphorylation by PDK-1, as indicated by a recent review [12], early work on purified PKC ζ from bovine kidney had shown allosteric activation by

acidic lipids [13,14]. The kinase function of the freed catalytic domain of PKC ζ (called CAT ζ) and other PKC isoforms has long been thought to be ‘constitutively active’, because it is divorced from the inhibitory regulatory domain, which includes a pseudosubstrate segment that occludes the phosphoacceptor-binding pocket [1–3]. Presumably phosphorylation of Thr⁴¹⁰ activates CAT ζ by inducing a conformational change, which repositions the catalytic groups involved in substrate interactions and phosphate transfer. PDK-1 is a major upstream effector kinase of PKC ζ and CAT ζ in mammalian cells because amino acid substitutions for Phe⁵⁷⁸ of the PDK-1 docking site markedly depressed Thr⁴¹⁰ phosphorylation and the activity of full-length PKC ζ and CAT ζ in transfected mammalian cells [9,15]. Treatment with foetal bovine serum strongly stimulated CAT ζ kinase activity and increased its pThr⁴¹⁰ (phosphothreonine) content in serum-starved HeLa cells [9]. Generally, full-length PKC ζ is active in proliferating cells and depressed in cell cultures that were deprived of growth factors [10,15–17].

Caspase processing of PKC ζ might activate its kinase function simply by untethering the regulatory domain, which contains an autoinhibitory pseudosubstrate motif, from the kinase domain. However, phosphorylation of the activation loop of the freed kinase domain, CAT ζ , is essential for the kinase function [9]. Therefore increased phosphorylation of Thr⁴¹⁰ could explain some or all of the increased kinase activity associated with the production of CAT ζ following the treatment of HeLa cells with TNF α (tumour necrosis factor α). In the present paper, we show that caspase processing of PKC ζ to CAT ζ markedly activates the kinase function without affecting the Thr⁴¹⁰ phosphorylation *in vitro* or in transfected HeLa cells. CAT ζ had a much shorter

Abbreviations used: Ac-DEVDal, acetyl-Asp-Glu-Val-Asp-aldehyde; CAT ζ , catalytic domain of PKC ζ ; CHX, cycloheximide; DAG, diacylglycerol; DTT, dithiothreitol; MBP, myelin basic protein; PDK-1, phosphoinositide-dependent kinase 1; PKC, protein kinase C; pThr, phosphothreonine; TNF α , tumour necrosis factor α ; zIEALal, benzyloxycarbonyl-Ile-Glu(O-t-butyl)-Ala-leucinal; zVAD-fmk, benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone.

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half-life in the cells than PKC ζ , and the TNF α treatment destabilized the CAT ζ protein by increasing its degradation by the ubiquitin–proteasome system. Our findings show for the first time that the phosphorylation of PKC ζ at the activation loop, which is necessary for catalytic competency, is insufficient to relieve the autoinhibition imposed by the regulatory domain.

MATERIALS AND METHODS

Reagents

Lactacystin was prepared as described [18] and generously provided by S. Omura. Acetyl-Ala-Leu-Leu-norleucinal, Ac-DEVDal (acetyl-Asp-Glu-Val-Asp-aldehyde), and the cell-permeant peptide derivatives, zIEALal (benzyloxycarbonyl-Ile-Glu (O-t-butyl)-Ala-leucinal) and zVAD-fmk (benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone), were from Bachem Bioscience (King of Prussia, PA, U.S.A.). Lactacystin and the peptide derivatives were dissolved in DMSO as 1000-fold concentrated stock solutions. Monoclonal antibodies (unconjugated and conjugated to horseradish peroxidase) against the V5 epitope tag were from Invitrogen (Carlsbad, CA, U.S.A.). Recombinant porcine TNF α was from R&D Systems (Minneapolis, MN, U.S.A.). The ε peptide (ERMRRPRKRGSGSVRRRV) was from BioSource International (Camarillo, CA, U.S.A.), and PDKtide (KTFCGTPEYLAPEVRREPRILSEEEQEMFRDFDYIADWC) (Upstate Biotechnology, Lake Placid, NY, U.S.A.). Lipids were from Avanti Polar Lipids (Alabaster, AL, U.S.A.), except *sn*-1,2-dipalmitoyl PtdIns(3,4,5) P_3 , which was from Calbiochem (San Diego, CA, U.S.A.).

Cell culture, plasmid constructs and transfection

HeLa and transformed HEK-293 cells (A.T.C.C. no. CRL-1573) cells were grown in Dulbecco's modified Eagle's medium containing 10% (v/v) foetal bovine serum, 100 units/ml penicillin G and 0.1 mg/ml streptomycin. LLC-PK $_1$ cells (A.T.C.C. no. CL-101) were grown in Alpha modification of Eagle's Medium containing 10% foetal bovine serum, 100 units/ml penicillin G and 0.1 mg/ml streptomycin. Cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO $_2$ and 95% air. HEK-293 cells were transfected by a modified calcium phosphate method [19]. HeLa cells were transfected with the previously described plasmid constructs, pcDNA3.1/GS human full-length PKC ζ (amino acids 1–592) or CAT ζ (amino acids 1–15 and 240–592) with C-terminal V5 and His $_6$ tags, using X-treme-GENE Q2 reagent as recommended by the manufacturer (Roche Molecular Biochemicals, Indianapolis, IN, U.S.A.) [6]. Transfection efficiencies were optimized with pcDNA3.1LacZ plasmid (Invitrogen) and histochemical staining with X-Gal, a β -galactosidase substrate [20]. HeLa cells were supplied with fresh medium containing 10% (w/v) foetal bovine serum the day after transfection. The following day, the cells were treated with recombinant porcine TNF α and/or CHX (cycloheximide), which were added directly to the culture medium.

Wild-type and mutant PKC ζ and CAT ζ plasmids have been previously described [6,9]. The PKC ζ/ι chimaera (codons 182–239 of PKC ζ are replaced by codons 178–234 of PKC ι , numbering from the initial ATG) was produced by PCR and restriction-digest subcloning. PKC ι cDNA was produced by PCR from total RNA from HeLa cells using forward (5'-TCGGATCCACC-ATGTCACACACGGTCGCAGGCGGCGGCA-3') and reverse (3'-GCGGCCGCGACACATTTCTTCTGCAGACATCAAAGA-GG-5') primers with *Bam*HI and *Not*I restriction sites, respectively. The PCR product was cloned with the pCR-Blunt vector

(Novagen, Madison, WI, U.S.A.), and subcloned by restriction digestion and ligation into pcDNA3.1V5His plasmid (Invitrogen). The PKC ι plasmid was used as a template for PCR with forward (5'-CTGCAGGACAATTGAATGTGGGCGGCATTC-3') and reverse (3'-CTGCAGCCCAAGCCCCTGAGAGATTTTGCCACT-TTCCCTGGTGTTC-5') primers, which include *Pst*I restriction sites that flank the segment of PKC ζ that will be replaced by the corresponding segment of PKC ι . The PCR product was digested with *Pst*I and was ligated into the PKC ζ plasmid described previously with C-terminal V5 and His $_6$ tags [6]. Each of the cDNA constructions was validated by sequencing using dye-terminator chemistry and an ABI Prism 377 automated sequencer.

Purified wild-type and mutant forms of PKC ζ with C-terminal V5 His $_6$ tags were metal-affinity-purified from transfected HEK-293 cells as previously described [9]. PDK-1 was affinity-purified from transfected human embryonic retinoblast 911 cells as described [9]. CAT ζ was affinity-purified from *Escherichia coli* as described [9]. Recombinant purified caspase 3 was prepared as described [21] and was generously provided by Dr Robert V. Talanian (BASF Bioresearch Corp., Worcester, MA, U.S.A.). The caspase reaction buffer contained 50 mM Hepes titrated to pH 7.5 with Tris, 1 mM DTT (dithiothreitol), 0.5 mM EDTA and 10% (v/v) glycerol.

Immunoprecipitation and kinase assays

Immunoprecipitation of epitope-tagged PKC ζ or CAT ζ with a monoclonal antibody against the V5 epitope tag (Invitrogen) and assay of immune-complex kinase activity using 40 μ M of ε peptide or MBP (myelin basic protein) as phosphoacceptor were performed essentially as previously described [6,9]. Briefly, transfected HeLa cells were lysed with a buffer containing 25 mM Tris/HCl, pH 7.5 (final), 1 mM EDTA, 0.5 mM EGTA, 0.1% (v/v) Nonidet P40, 0.5 mM DTT, 20 mM β -glycerophosphate, 0.1 mM sodium orthovanadate, 1 mM Pefabloc SC and Pefabloc PLUS (Roche Molecular Biochemicals), 0.025 mM acetyl-Ala-Leu-Leu-norleucinal and 2 μ g/ml leupeptin. The lysate was clarified by centrifugation at 16 000 *g* for 15 min at 4 °C. The protein concentration of the supernatant was measured by the BCA (bicinchoninic acid) method (Pierce, Rockford, IL, U.S.A.). A sample of the lysate (usually 0.1 mg) was diluted to 1 ml with lysis buffer, which contained 0.05% (v/v) Nonidet P40. Immunoprecipitation was performed with 0.5 mg of anti-V5 monoclonal antibody (Invitrogen) for 3 h at 4 °C, and Protein A/G–agarose (Santa Cruz Biotechnologies, Santa Cruz, CA, U.S.A.) (20 μ l of 25% packed agarose) was present during the last 1 h. Immunoprecipitates were rinsed twice with lysis buffer containing 0.3 M NaCl and 0.05% (v/v) Nonidet P40, twice with the same buffer without NaCl, and once with kinase assay buffer (no ATP or phosphoacceptor).

Each assay (30 μ l) contained 25 mM Hepes/Tris, pH 7.4, 20 mM β -glycerophosphate, 10 mM MgCl $_2$, 2 mM DTT, 0.1 mM sodium orthovanadate, 20 μ M ATP, 1–5 μ Ci of [γ - 32 P]ATP (ICN Biochemicals, Aurora, OH, U.S.A.), 40 μ M ε peptide or 5 μ g of MBP and a PKC ζ immune complex or affinity-purified PKC ζ . Lipids (100 μ M *sn*-1,2-dioleoyl-*sn*-glycero-3-phosphocholine, 100 μ M *sn*-1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine, 10 μ M *sn*-1,2-dipalmitoyl PtdIns(3,4,5) P_3 and 10 μ M ceramide) were included in some assays (results not shown). Liposomes (5 $\times$ concentrated) were prepared by evaporation of the organic solvent in which the lipids were dissolved with a stream of N $_2$, suspension by vortex mixing with 10 mM Hepes/Tris, pH 7.4, containing 1 mM EGTA, and sonication for 20 min at room temperature (21 °C) using a bath

sonicator (Branson 3210, Branson Ultrasonics, Danbury, CT, U.S.A.). Kinase assays were started by adding the complete kinase assay buffer to the immune complex or by adding a sample of the caspase 3 reaction to kinase assay buffer, which contained 1 μ M of the caspase 3 inhibitor, Ac-DEVDal. After 30 min at 30 °C, the reactions were stopped by the addition of 5 μ l of 0.9 M phosphoric acid and placed on ice until a 20 μ l sample was spotted on to a 2.1 cm diameter phosphocellulose P81 disc (Whatman, Fairfield, NJ, U.S.A.). Discs were rinsed four times with 0.15 M phosphoric acid, and the amount of 32 P-labelled peptide bound to the disc was determined by scintillation counting. [32 P]MBP was quantified by autoradiography after SDS/PAGE as described [6].

PDK-1 activity was assayed with PDKtide as the phospho-acceptor peptide [22]. Each reaction (25 μ l) contained 25 mM Hepes, adjusted to pH 7.4 with Tris, 10 mM MgCl_2 , 0.1 mM sodium orthovanadate, 2 mM DTT and 20 mM β -glycerophosphate. In addition, each reaction contained 40 μ M PDKtide and liposomes containing 100 μ M *sn*-1,2-dioleoyl-*sn*-glycero-3-phosphocholine, 100 μ M *sn*-1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine, 10 μ M *sn*-1,2-dipalmitoyl PtdIns(3,4,5) P_3 and 20 μ M ATP (approx. 1 μ Ci of [γ - 32 P]ATP). Reactions were started by adding enzyme and were stopped after 30 min at 30 °C by adding 5 μ l of 0.9 M phosphoric acid. Reactions were placed on ice until a sample was spotted on to a 2.1 cm diameter phosphocellulose P81 disc and rinsed as described above.

Western blot analyses

A horseradish-peroxidase-conjugated anti-V5 antibody (Invitrogen) was used for Western blot analysis of immunoprecipitates from HeLa cells transfected with epitope-tagged PKC ζ or CAT ζ . Western blot analysis was performed essentially as previously described [6,23]. Briefly, proteins were fractionated by SDS/PAGE (10 % gel), transferred electrophoretically on to a PVDF membrane (Millipore, Billerica, MA, U.S.A.). The membrane was blocked for 1 h at room temperature with 5 % (w/v) non-fat dried milk in Tris-buffered saline, which contained 8 g/l NaCl, 0.2 g/l KCl and 3 g/l Tris, and was adjusted to pH 7.4 with HCl. Following the incubation with horseradish-peroxidase-conjugated first or second antibody, the membrane was rinsed with Tris-buffered saline containing 0.05 % (w/v) Tween 20, and incubated with a chemiluminescent substrate [23]. Some membranes were immunostained with a rabbit polyclonal antibody (#2291 Cell Signaling Technology, Beverly, MA, U.S.A.) that specifically recognizes the pThr-Phe-Cys-Gly-Thr motif. Western blots are representative of at least three independent experiments.

RESULTS

TNF α induces caspase processing of wild-type PKC ζ to CAT ζ and increases immune complex ζ kinase activity

Treatment of transfected HeLa cells with TNF α plus CHX, which is known to activate caspases and induce apoptosis in mammalian cells, evoked the production of CAT ζ (Figure 1). CAT ζ is known to be produced by caspase processing of endogenous or ectopically expressed PKC ζ , which is cleaved chiefly, but not exclusively, at Asp²¹⁰ and Asp²³⁹ [6]. The TNF α treatment (5 h) converted almost all PKC ζ into CAT ζ and increased immune-complex kinase activity approx. 3-fold (Figure 1, lanes 1 and 2). Immune-complex kinase activity was specific for ectopic PKC ζ because immune complexes of Thr⁴¹⁰ $\rightarrow$ Ala (T410A) mutant PKC ζ from untreated cells were essentially devoid of kinase activity (Figure 1), as previously reported [9].

Figure 1 Caspase processing and activation of PKC ζ following the induction of apoptosis by TNF α plus CHX in HeLa cells transfected with wild-type or mutant PKC ζ

Wild-type, T410A mutant PKC ζ or the PKC ζ/ι chimaera was expressed in HeLa cells (upper panel). The indicated cultures were treated with 50 ng/ml TNF α plus 10 μ g/ml CHX for 5 h. The cells were lysed, and PKC ζ was immunoprecipitated with antibody to the V5 epitope. Immunoprecipitates were assayed for kinase activity with ϵ peptide as substrate and subjected to Western blot analysis with horseradish-peroxidase-conjugated antibody against the V5 epitope. Kinase activities (lower panel) are means $\pm$ S.E.M. ($n = 3$). * $P < 0.05$ (treated versus untreated control), as determined by Student's t test.

Note that the C-terminal fragments of the T410A mutant CAT ζ failed to accumulate following the TNF α /CHX treatment, even though the T410A mutant PKC ζ was completely converted into T410A mutant CAT ζ proteins (Figure 1, lane 4). Results, which are presented below (Figure 1, lower panel), indicate that T410A mutant CAT ζ protein is particularly short-lived in the TNF α -treated cells.

TNF α fails to induce caspase processing or activation of the PKC ζ/ι chimaera

In contrast with PKC ζ , the PKC ζ/ι chimaera was neither processed to CAT ζ nor activated by TNF α treatment (Figure 1, lanes 5 and 6). The chimaera was constructed by replacement of the approx. 60-residue unique segment of PKC ζ by the corresponding segment of PKC ι . These two PKC isoforms are highly homologous (71 % identity) and differ mostly in the sequence of the initial 15 amino acids and the last 60 amino acids of the regulatory domain, which contains the caspase-processing sites [6]. The chimaera was constructed because replacement of Asp²¹⁰, Asp²²² and Asp²³⁹ with alanine failed to prevent caspase processing of PKC ζ to CAT ζ in HeLa cells, and because PKC ι is not processed by caspases [8]. The specific kinase activity of the chimaera was similar to that of wild-type PKC ζ (Figure 1, lanes 1 and 5). These findings support the hypothesis that the conversion of PKC ζ into CAT ζ is required for the increased kinase activity following TNF α treatment.

Figure 2 Time course of caspase processing and activation of PKC ζ following the induction of apoptosis by TNF α plus CHX in transfected HeLa cells

HeLa cells were transfected with PKC ζ and were treated with 50 ng/ml TNF α plus 10 μ g/ml CHX for the indicated intervals. The cells were lysed, and PKC ζ was immunoprecipitated with antibody against the V5 epitope (upper panel). Immunoprecipitates were assayed for kinase activity with 40 μ M ϵ peptide as substrate (middle panel). After the kinase assay, samples of each immunoprecipitate were subjected to Western blot analysis with a rabbit polyclonal antibody that recognizes pThr⁴¹⁰ (P-Thr⁴¹⁰) of PKC ζ (anti-T*FCGT) (lower panel). After stripping and demonstrating that the stripped membrane had no detectable horseradish peroxidase activity, the membrane was reprobed with horseradish-peroxidase-conjugated antibody against the V5 epitope. Kinase activities are means $\pm$ S.E.M. ($n=3$; * $P<0.05$) (treated versus untreated control), as determined by Student's t test.

Figure 2 shows the time course of the conversion of PKC ζ into CAT ζ in TNF α -treated HeLa cells. After a 1 h lag, treatment with TNF α for 2–5 h evoked the progressive conversion of the PKC ζ into CAT ζ (Figure 2, upper panel). The time-dependence of the increase in immune-complex kinase activity was similar to that of the accumulation of the CAT ζ protein (Figure 2, upper and middle panel). There was no significant increase in kinase activity or CAT ζ protein at 0.5 or 1 h, and most of the increase in kinase activity and CAT ζ protein occurred between 1 and 3 h of the TNF α treatment (Figure 2, middle panel). The maximal increase in kinase activity was approx. 3-fold.

Treatment with TNF α /CHX had no effect on Thr⁴¹⁰ phosphorylation of PKC ζ or CAT ζ

Previous reports have shown that phosphorylation of the activation loop of PKC ζ at Thr⁴¹⁰ is necessary and sufficient to activate the kinase function [10,11,15]. To determine whether or not TNF α treatment affected the pThr⁴¹⁰ content of PKC ζ , samples of the immune complex were subjected to Western blot analysis with an antibody that specifically recognizes pThr⁴¹⁰ (Figure 2, bottom panel). Western blot with the anti-pThr⁴¹⁰ antibody showed that the TNF α -induced changes in the immunostaining intensities of

Figure 3 *In vitro* processing of purified PKC ζ by recombinant caspase 3 is sufficient to activate the kinase function

C-terminal V5 His₆-tagged PKC ζ was affinity-purified from transfected HEK-293 cells and was incubated at 30 °C for the indicated intervals with or without 90 ng of recombinant caspase 3 (20 μ l per reaction). Ac-DEVDal (1 μ M) was present where indicated to inhibit caspase 3. The caspase reactions were stopped by adding 10 μ l of the kinase reaction buffer, which contained 1 μ M Ac-DEVDal. Kinase activity was assayed by determining the rate of ϵ peptide (40 μ M) phosphorylation at 30 °C. Samples (10 μ l) of the kinase reaction were used for quantifying [³²P] ϵ peptide and SDS/PAGE. Kinase activities are means $\pm$ S.E.M. ($n=3$; * $P<0.05$) (treated versus untreated control), as determined by Student's t test. Duplicate samples of each kinase reaction were subjected to Western blot analysis with antibody against the V5 epitope or the antibody that recognizes pThr⁴¹⁰ (P-Thr⁴¹⁰) of PKC ζ (anti-T*FCGT).

PKC ζ and CAT ζ bands were similar to those observed with the antibody against the V5 epitope tag (Figure 2, upper and lower panels, lanes 1–3). Densitometric analysis of the full-length PKC ζ bands showed that the ratio of pThr⁴¹⁰ to V5 epitope immunostaining did not change significantly from 0 to 4 h of TNF α treatment. The mean ratio was 1.41 ± 0.10 ($n=6$). Similarly, the ratio of the pThr⁴¹⁰ to V5 immunostaining of the more prominent of the two CAT ζ bands did not change significantly from 2 to 5 h of TNF α treatment (0.97 ± 0.11 ; $n=4$). Therefore TNF α treatment did not markedly affect the phosphothreonine content of PKC ζ or CAT ζ , which were previously shown to be largely phosphorylated at this residue, unless the cells were serum-deprived [9]. These results indicate that the increased kinase activity that accompanied the conversion of PKC ζ into CAT ζ occurred without a marked change in the pThr⁴¹⁰ content of CAT ζ .

Activation of recombinant purified PKC ζ by caspase 3 *in vitro*

To determine whether or not caspase processing was sufficient to activate the specific kinase activity of PKC ζ , recombinant PKC ζ was purified from transfected HEK-293 cells and was incubated with recombinant purified caspase 3 from bacteria. Figure 3

shows that incubation with caspase 3 for 7.5–60 min progressively increased the specific activity of the kinase. Maximal stimulation was approx. 5-fold. Addition of 1 μ M Ac-DEVDal, a caspase 3 inhibitor, during the incubation with caspase 3 abolished the increase in kinase activity (Figure 3). Western blot analysis with antibody against the V5 epitope showed that caspase 3 converted PKC ζ into CAT ζ , and that the caspase inhibitor almost abolished the production of CAT ζ (Figure 3). Previously, we showed that recombinant caspase 3 chiefly processed PKC ζ at Asp²¹⁰ and Asp²³⁹, which explains the production of slower and faster mobility CAT ζ bands respectively [6]. The *in vitro* data indicate that caspase 3 processing of purified PKC ζ is sufficient to activate the kinase function.

***In vitro* caspase 3 processing of PKC ζ or CAT ζ had no effect on Thr⁴¹⁰ phosphorylation**

Because PDK-1 forms a stable complex with PKC ζ , it is possible that our PKC ζ preparation contained PDK-1. Accordingly, it is possible that caspase 3 processing of PKC ζ *in vitro* affected the phosphothreonine content of the kinase. The following observations provide evidence that there was no change in pThr⁴¹⁰ following the processing of PKC ζ to CAT ζ .

First, densitometric analysis of the Western blots showed that there was no significant change in the ratios of pThr⁴¹⁰ to V5 epitope staining following the conversion of PKC ζ into CAT ζ *in vitro* (Figure 3). The mean ratio of full-length PKC ζ (0–60 min of treatment) was 0.92 ± 0.07 ($n = 5$) and that of the slower mobility CAT ζ bands was 0.85 ± 0.06 ($n = 4$). Secondly, there was no detectable PDK-1 activity in the affinity-purified PKC ζ , as determined using PDKtide as a substrate (results not shown). Affinity-purified PDK-1 from human embryonic retinoblast 911 cells was highly active towards PDKtide and served as a positive control. Thirdly, the affinity-purified PKC ζ used for *in vitro* caspase processing appears to be maximally phosphorylated at Thr⁴¹⁰. Thus treatment of the purified PKC ζ from HEK-293 cells with purified recombinant PDK-1 had no effect on pThr⁴¹⁰ content, as determined by Western blot analysis (Figure 4). As a positive control, the treatment with PDK-1 phosphorylated Thr⁴¹⁰ of recombinant purified CAT ζ from *E. coli* (Figure 4), as previously reported [9]. The PDK-1 treatment activated the kinase activity of the recombinant CAT ζ , as previously reported [9], but had no effect on the kinase activity of PKC ζ from HEK-293 cells (results not shown). These findings agree with those of Balendran et al. [15], which showed that PKC ζ was highly active and phosphorylated at Thr⁴¹⁰ following expression in HEK-293 cells, and suggest that PKC ζ from HEK-293 cells is essentially phosphorylated stoichiometrically at Thr⁴¹⁰.

Stability of PKC ζ and wild-type and mutant CAT ζ proteins

The half-lives of PKC ζ , PKC ι , the ζ/ι chimaera, CAT ζ and mutant CAT ζ were compared by quantifying the steady-state cellular levels of the proteins by Western blot analysis following the blockade of protein synthesis with CHX. Figure 5 shows representative Western blots, and a summary of the half-life data from multiple experiments is shown in Table 1. CAT ζ had a half-life of approx. 5 h in the absence of TNF α and 2.5 h in its presence (Figure 5B and Table 1). In contrast with CAT ζ , full-length PKC ζ , PKC ι and the chimaera were stable and failed to decrease significantly even after a 9 h blockade of protein synthesis (Figure 5A). It was not possible to measure the stability of PKC ζ in the presence of TNF α because it is converted into CAT ζ . Therefore the stability of PKC ι and the chimaera, which are not processed

Figure 4 Lack of effect of treatment with PDK-1 on the pThr⁴¹⁰ content of PKC ζ from HEK-293 cells

Purified PKC ζ from HEK-293 cells or CAT ζ from *E. coli* was treated with or without recombinant purified PDK-1 as indicated. Following the treatment, samples were subjected to Western blot analysis with the antibody that specifically recognizes pThr⁴¹⁰ (P-Thr⁴¹⁰) of PKC ζ (anti-T*FCGT). To compare the relative amounts of PKC ζ and CAT ζ proteins, the membrane was stripped and reprobed with a polyclonal antibody against the extreme C-terminal segment of PKC ζ (C-20, Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.).

by caspases (Figure 1), were determined in the presence of TNF α . Treatment with TNF α plus CHX for 4 or 5 h failed to decrease the levels of the PKC ι or the chimaera proteins, which indicates that they are relatively stable (Figure 5B; and results not shown). The duration of the treatment was limited to 5 h because the TNF α treatment rapidly induced apoptosis, which would confound half-life determinations at longer intervals. Interestingly, the T410A mutant CAT ζ had a half-life of only 1.2 h in the TNF α -treated cells (Figure 5B and Table 1). Substitution of a glutamic residue for Thr⁴¹⁰ of CAT ζ (T410E) produced a protein with a half-life similar to that of wild-type CAT ζ (Figure 5B and Table 1). The negative charge of the glutamic residue may partially mimic that of pThr⁴¹⁰, which apparently increases the stability of the CAT ζ protein. The T410E mutant CAT ζ had very low, but significant, kinase activity, whereas the T410A mutant lacked detectable kinase activity [9].

Previously, we observed that the PDK-1 docking site mutant, F578A CAT ζ , had no detectable pThr⁴¹⁰ or kinase activity following expression in HeLa cells [9], which we have confirmed (results not shown). Interestingly F578A CAT ζ had a half-life similar to wild-type CAT ζ in the TNF α -treated cells (Figure 5B and Table 1). This finding suggests that the lack of pThr⁴¹⁰ is not sufficient to destabilize CAT ζ and suggests that destabilization depends on additional factors, such as PDK-1 binding.

Instability of endogenous CAT ζ protein in renal epithelial cells

Western blot analysis of the LLC-PK₂ line of porcine kidney epithelial cells showed that the untreated cells had detectable CAT ζ ,

Figure 5 Comparison of the stability of PKC ζ, PKC ι, the PKC ζ/ι chimaera, and wild-type and mutant CAT ζ proteins in transfected HeLa cells

HeLa cells were transfected with pcDNA3.1 plasmid encoding the indicated PKC ζ construct with C-terminal V5 and His₆ tags. Following treatment with 10 μg/ml CHX alone (**A**) or with 50 ng/ml TNFα plus 10 μg/ml CHX (**B**), the cells were lysed, and a sample of the lysate was subjected to Western blot analysis with horseradish-peroxidase-conjugated antibody against the V5 epitope and chemiluminescent detection. X-ray films were scanned and analysed by volume densitometry (BioRad GS-670). WT, wild-type.

Table 1 Summary of half-life determinations of PKC ζ, PKC ι and the chimaera, and wild-type and mutant CAT ζ in the presence or absence of TNFα

Half-lives were determined by non-linear regression fitting to a single exponential decay equation (InPlot 3.1, GraphPad Software, San Diego, CA, U.S.A.). Values are means ± S.E.M. The half-lives of PKC ζ, PKC ι and the chimaera were not determined because the proteins were essentially stable for the duration of the treatments (9 h with CHX alone or 5 h with TNFα plus CHX).

Plasmid	TNFα	Half-life (h)
Wild-type PKC ζ	No	> 9
Wild-type PKC ι	No	> 9
Wild-type PKC ζ/ι	Yes	> 5
PKC ζ/ι chimaera	Yes	> 5
Wild-type CAT ζ	No	4.9 ± 0.6 (n=5)
Wild-type CAT ζ	Yes	2.5 ± 0.2 (n=11)
T410A mutant CAT ζ	Yes	1.2 ± 0.1 (n=11)
T410E mutant CAT ζ	Yes	3.1 ± 0.4 (n=3)
F578A CAT ζ	Yes	2.2 ± 0.3 (n=7)

which was relatively small in amount compared with the full-length atypical PKC band (Figure 6A, short exposure). Because the antibody does not distinguish between the two atypical PKC isoforms, it is uncertain whether the approx. 70 kDa band is PKC ζ or a combination of the two atypical PKC isoforms. Nevertheless, the approx. 40 kDa band is CAT ζ because PKC ι is not processed by caspases, as reported previously and as shown above (Figure 1; and results not shown). Moreover, treatment of the cells with TNFα/CHX markedly increased the level of the 40 kDa CAT ζ band and produced the slower mobility CAT ζ

Figure 6 Instability of endogenous CAT ζ proteins in porcine kidney epithelial cells

(**A**) Confluent cultures of LLC-PK₂ cells were treated for 3 h with 50 ng/ml TNFα plus 10 μg/ml CHX and/or the indicated concentration of zVAD-fmk, which was added 1 h before the addition of TNFα plus CHX. Lanes 2 and 3 are from duplicate cultures treated with TNFα plus CHX. At 100 μM zVAD-fmk, the production of CAT ζ proteins by TNFα plus CHX was abolished. The upper panel (Short Exposure) shows the relative amount of PKC ζ/ι and CAT ζ bands. The lower panel (Long Exposure) shows the effects of the treatments on CAT ζ bands. (**B**) Treatment of the cells with zVAD-fmk (100 μM) for 0.5, 1, 2, or 4 h depleted endogenous CAT ζ. DMSO, which was used to dissolve zVAD-fmk, had no effect on the level of the CAT ζ protein at 0.1% (v/v) when present for 1, 2 or 4 h. Western blots are representative of at least three independent experiments.

species (Figure 6A, long exposure, lanes 2–6). The production of the two CAT ζ bands is characteristic of CAT ζ produced from transfected PKC ζ (Figures 1 and 2) or endogenous PKC ζ [6,9].

To determine whether endogenous CAT ζ was a stable or unstable protein, the cells were treated with the cell-permeant general caspase inhibitor, zVAD-fmk, to terminate the production of CAT ζ. Treatment of the cells with 100 μM, but not 1 μM, zVAD-fmk for 4 h caused the disappearance of CAT ζ (Figure 6A, long exposure, lanes 8 and 9). Note that zVAD-fmk at 100 μM (Figure 6A, long exposure, lane 7), but not at the lower concentrations, abolished CAT ζ production by the TNFα/CHX treatment (Figure 6A, long exposure). Figure 6(B) shows that treatment with zVAD-fmk for 0.5, 1, 2 or 4 h essentially depleted all detectable endogenous CAT ζ from the renal epithelial cells.

Figure 7 Proteasome inhibition stabilized CAT ζ and T410A mutant CAT ζ proteins and CAT ζ kinase activity following the treatment of HeLa cells with TNF α and CHX

HeLa cells were transfected with CAT ζ (**A**) or T410A mutant CAT ζ (**B**) with C-terminal V5 and His₆ tags. The indicated cultures were incubated for 15 min with a proteasome inhibitor, 20 μ M lactacystin (Lact) or zIEALal, before the addition of 50 ng/ml TNF α or 10 μ g/ml CHX, or both. After 4 h, the cells were lysed, and a 20 μ g sample of the lysate (**A**) and (**B**) was subjected to Western blot analysis with horseradish-peroxidase-conjugated antibody against the V5 epitope. CAT ζ was immunoprecipitated with antibody against the V5 epitope (**A**), and immune-complex kinase activity was assayed with 5 μ g MBP as substrate per assay (30 μ l). Data are representative of at least three independent experiments.

zVAD-fmk (100 μ M) was dissolved in DMSO, which by itself at 0.1 % had no effect on the level of CAT ζ in the cells (Figure 6B). Because the spontaneously produced, endogenous CAT ζ in the renal epithelial cell line essentially disappeared in 0.5 h (Figure 6B), it was even less stable than CAT ζ in transfected HeLa cells (Figure 5 and Table 1).

Proteasome inhibitors stabilize CAT ζ protein and kinase activity

Treatment with TNF α potentiated the loss of CAT ζ protein and kinase activity following the blockade of protein synthesis (Figure 7A). Selective proteasome inhibitors, lactacystin or zIEALal, largely prevented the loss of CAT ζ protein and kinase activity following the treatment of the cells with TNF α plus CHX (Figure 7A, lanes 4, 6 and 8). The treatment with either of the proteasome inhibitors abolished proteasome peptidase activity, which was assayed as described previously [6] (results not shown). Proteasome blockade largely prevented the loss of the unstable T410A mutant of CAT ζ during the treatment with TNF α plus CHX (Figure 7B, lanes 2, 3 and 4). Previously, the accumulation of multi-ubiquitinated forms of CAT ζ were shown to accumulate in mammalian cells following proteasome blockade [6]. These findings indicate that the ubiquitin-proteasome system is largely responsible for the degradation of wild-type CAT ζ and the less stable T410A mutant CAT ζ .

DISCUSSION

The present paper concerns two mechanisms that regulate the kinase function of atypical PKC ζ . The first is the relief of the autoinhibitory state by caspase processing which untethers the regulatory and kinase domains. The second is the disassembly of the freed kinase domain by the ubiquitin-proteasome system. Allosteric activation of the conventional and novel PKC isoforms by calcium and/or DAG is well known to occur by relief of the autoinhibitory state imposed by the regulatory domain [1-3]. However, the activity of atypical PKCs does not depend on calcium or DAG, and phosphorylation of the activation loop is sufficient to activate the kinase function of PKC ζ [1-3,10,11]. Therefore it has remained unclear whether atypical PKCs are subject to allosteric regulation, for example, by a lipid, such as 3-phosphoinositides or ceramide [14,24-26], or whether phosphorylation of the activation loop is sufficient to maximally activate the kinase function. The present findings indicate that activation-loop phosphorylation (Thr⁴¹⁰ of PKC ζ) is not sufficient to maximally activate the kinase. Thus *in vitro* processing of purified recombinant PKC ζ at the hinge region between the regulatory and kinase domains by caspase 3 produced a 5-fold increase in the specific activity of the kinase (Figure 3). Essentially, all Thr⁴¹⁰ of the recombinant PKC ζ , which was affinity-purified from transfected HEK-293 cells, appeared to be phosphorylated (Figure 4). This finding agrees with previous reports that had suggested that most of the Thr⁴¹⁰ of PKC ζ is phosphorylated following ectopic expression in HEK-293 cells, with the exception of serum-deprived cultures [9,15]. It was essential to address the status of Thr⁴¹⁰ because the kinase function of the freed kinase domain (CAT ζ) depends on its phosphorylation like that of the holoenzyme [9]. These findings indicate that *in vitro* caspase processing of recombinant PKC ζ , which appeared to be fully phosphorylated at Thr⁴¹⁰, was sufficient to activate the kinase function *in vitro*.

Our results on intracellular caspase processing of PKC ζ in transfected HeLa cells support the idea that untethering of the regulatory and kinase domains is sufficient to activate the kinase function. Thus treatment of the cells with TNF α evoked the production of CAT ζ and increased immune-complex kinase activity 3-fold with no apparent increase in Thr⁴¹⁰ phosphorylation (Figure 2). Studies of a PKC ζ/ι chimera, which lacked the caspase-processing sites, confirmed that proteolysis at the hinge region between the two domains was essential for activation of the kinase function following the TNF α treatment (Figure 1). The chimera was used for this experiment because the two atypical PKC isoforms are highly homologous (71 % identity), having sequences that diverge chiefly in the last 60-amino-acid segment of the regulatory domain of PKC ζ , which contains several caspase-processing sites ([9]; and results not shown). The corresponding segment of PKC ι lacks consensus caspase-processing sites, and PKC ι is not a caspase substrate [6,8]. Interestingly, PKC ι , but not PKC ζ , protected human K562 leukemia cells from drug-induced apoptosis [8]. Although PKC ζ appears to promote cell survival, at least in part via activation of NF- κ B (nuclear factor- κ B) [27], little is known about the protein substrates and signalling events transduced by either PKC ζ or CAT ζ .

The present findings indicate that allosteric activation is required to maximally activate PKC ζ . Hence PKC ζ , which appeared to be stoichiometrically phosphorylated at the activation loop, had approx. 20 % of the maximal kinase activity, which was produced by untethering the regulatory and kinase domains. We have observed that phosphatidylserine alone stimulated the kinase activity of full-length PKC ζ 4-fold, but had no effect on CAT ζ activity (results not shown). The stimulatory effect of

phosphatidylserine on recombinant PKC ζ is similar to that described by Nakanishi and Exton [13] for PKC ζ purified from bovine kidney. The zinc-finger motif of the regulatory domain, which is absent from CAT ζ , probably mediates the interaction with phosphatidylserine-containing membrane surfaces [3]. Additionally, we have observed that PtdIns(3,4,5) P_3 stimulated recombinant PKC ζ similarly to phosphatidylserine, and phosphatidylcholine diminished the stimulatory effects of the anionic lipids (results not shown), which agrees closely with the phospholipid dependence of purified renal PKC ζ [14]. PKC subtypes, other than atypical PKCs, have two zinc-finger motifs, one of which binds DAG [1–3]. DAG binding in turn confers remarkable selectivity for the phosphatidyl-L-serine headgroup [28]. Although more work is needed to characterize the specificity of the interaction of recombinant PKC ζ with lipids, the present findings show that it is strongly autoinhibited and it is subject to allosteric regulation.

Another striking consequence of the caspase processing of PKC ζ , in addition to the activation of the kinase function, was the dramatic destabilization of the protein. Both spontaneously produced, endogenous CAT ζ in renal epithelial cells and ectopically expressed CAT ζ in HeLa cells were relatively short-lived compared with full-length atypical PKC ζ or ι (Figures 5 and 6). In contrast with full-length PKC ζ or ι , which were stable for at least 9 h in transfected HeLa cells, CAT ζ had a half-life of approx. 5 h in the absence of TNF α and 2.5 h in its presence (Figure 5 and Table 1). Interestingly, the T410A mutant CAT ζ had a half-life of only 1.2 h (Figure 5 and Table 1). Thus the treatment of the cells with TNF α converted essentially all of the T410A PKC ζ into T410A CAT ζ , but it failed to accumulate because it was so unstable (Figure 1). A negative charge at residue 410, either from phosphothreonine or by replacement with a glutamic residue, seems to stabilize CAT ζ , although other factors, such as PDK-1 docking, also seem to affect the turnover of the CAT ζ protein (Figure 5 and Table 1). Proteasome blockade almost prevented the loss of CAT ζ proteins from the cells, which was evoked by TNF α (Figure 7). We have previously demonstrated the accumulation of multi-ubiquitinated PKC ζ and CAT ζ proteins in mammalian cells following proteasome blockade [6]. The observation that the T410A mutant CAT ζ was less stable than the T410E mutant (Figure 5 and Table 1) agrees with the proposal that dephosphorylation of PKC α predisposes it to degradation by the ubiquitin–proteasome system [23,29].

PKC ζ has two especially prominent PEST (Pro-Glu-Ser-Thr) segments (amino acids 183–198 and 198–219), as previously noted [29]. PEST segments contain at least one proline, glutamic or aspartic, and serine or threonine residue, and are flanked by a lysine, arginine or histidine residue [30]. Clearly, neither of the two prominent PEST segments are sufficient to destabilize either PKC ζ or the PKC ζ/ι chimera because both of these proteins were relatively stable, in contrast with CAT ζ , which lacks both PEST segments and was short-lived both in the presence and absence of TNF α (Figure 5 and Table 1). A recent report by Okuda et al. [31] showed that overexpression of the von Hippel–Lindau tumour suppressor protein could increase the ubiquitination of PKC λ in cotransfected cells. The von Hippel–Lindau protein is the target protein recognition component of a RING (really important new gene) finger ubiquitin–protein ligase, which transfers ubiquitin to HIF (hypoxia-inducing factor) [32]. However, the regulatory domain of PKC λ (rodent PKC ζ) was essential for the increased ubiquitination [31]. Therefore CAT ζ is not expected to be a substrate for the von Hippel–Lindau ubiquitin–protein ligase. Further studies are needed to identify the ubiquitin–protein ligase for CAT ζ and to determine whether or not dephosphorylation of Thr⁴¹⁰ increases its susceptibility to ubiquitination.

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