Phosphorylation is required for PMA- and cell-cycle-induced degradation of protein kinase C δ

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Classical and novel protein kinase C (PKC) isoforms are down-regulated as a result of chronic activation by certain tumour promoters and physiological stimuli; however, the mechanisms leading to down-regulation are not fully understood. In the present study, we have studied the PMA ('TPA')-induced degradation of PKC δ in NIH 3T3 cells under culture conditions where PKC δ displays cell-cycle-dependent down-regulation. In contrast with previous studies, a hyperphosphorylated form of this PKC isoform, promoted by calyculin A, was rapidly degraded

in PMA-treated cells. Similarly, the presence of calyculin A enhanced the down-regulation of PKC δ observed on G₁/S-phase progression through the cell cycle. Analysis of phosphorylation-site mutants indicated that the T-loop Thr⁵⁰⁵ phosphorylation site was critical for induced degradation.

Key words: down-regulation, dephosphorylation, proteasome, protein phosphatase.

INTRODUCTION

Protein kinase C (PKC) isoforms are involved in a diverse range of intracellular functions. This superfamily of lipid-activated serine/threonine kinases are divided into three subgroups according to their cofactor requirements: these include the classical PKCs, α , β I, β II and γ ; the novel PKCs, δ , ϵ , η and θ ; and the atypical PKCs, ζ / ι [1]. Chronic activation of classical and novel PKC isoforms, elicited by phorbol esters such as PMA ('TPA'), leads to their down-regulation. Since PKCs are one of the major receptor classes for phorbol esters [2], the long-term biological effects of these tumour promoters may arise from the combined effects of activation and down-regulation of PKC (alongside other receptors). The down-regulation of the different PKC isotypes may therefore play an important role in cell proliferation.

PKC δ is a ubiquitously expressed isoform that appears to be an important regulator of cell growth and cell-cycle progression [3,4]. In any given cell type, the different members of the PKC gene family can act either negatively or positively to regulate critical cell-cycle transitions, particularly for cell-cycle entry and exit and at G₁ and G₂ checkpoints [5]. For instance, PKC δ has been associated with anti-proliferative properties in many cell types. It is known to inhibit growth and induce differentiation in fibroblasts [3,6]. PKC δ -mediated cell-cycle delay in endothelial cells involves an increase in the cyclin-dependent kinase inhibitors p21^{cip1} and p27^{kip1} and the reduced expression of D1 and E cyclins [7,8]. Studies in Swiss 3T3 cells suggest that the down-regulation of PKC δ is under the control of the cell cycle [9,10]. Together, these lines of evidence point to the importance of the down-regulation of PKC δ in cell-cycle progression.

The control of many key cell-cycle regulators occurs through their phosphorylation and selective degradation via the ubiquitin/proteasome pathway. Proteins to be degraded by ubiquitin-mediated proteolysis are recognized by protein–ligase complexes, which allows the conjugation of ubiquitin to the substrate protein and subsequently targets it for degradation by the 26 S proteasome [11,12]. Ubiquitination is not only involved

in cell-cycle control, but is well known to play a role in many cellular functions, such as endocytosis, signal transduction, DNA repair and apoptosis [13]. The ubiquitin/proteasome pathway has been proposed as the major route of degradation for PKCs. Ubiquitination of PKC α , δ and ϵ have been detected in rat 3Y1 cells upon prolonged activation by phorbol esters [14]. Likewise, ubiquitination of activated PKC ζ in HeLa cells [15] and ubiquitination of activated PKC η in baby hamster kidney cells [16] has been observed. Interestingly, pVHL (protein encoded by the Von Hippel-Lindau tumour suppressor gene) has been shown to be involved in the ubiquitination of PKC λ / ι [17]. Whether the down-regulation of classical and novel PKCs (and possibly atypical PKCs) is mediated by a general regulatory mechanism, or rather by isotype-specific mechanisms, remains to be determined. In the present study, we have investigated the down-regulation of PKC δ in the context of its cell-cycle-dependent behaviour. The evidence indicates that PKC isoform-specific mechanisms exist and that for PKC δ degradation under these conditions requires its phosphorylation.

EXPERIMENTAL

Cell culture and transfection

NIH 3T3 mouse fibroblasts were maintained in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) foetal calf serum (FCS). Cells were passaged three times a week by detachment with 0.25% (v/v) trypsin/versene and seeded at 2×10^5 cells in 35-mm-diameter plates or at 5×10^5 cells in 100-mm-diameter plates. Cells (35- or 100-mm diameter plates) were transfected using LIPOFECTAMINETM reagent (Gibco BRL) with 1–5 μ g of plasmid DNA [myc-PKC δ in pEflink or green fluorescent protein (GFP)–PKC δ and mutants in pEGFPC3] for between 6–18 h. Following transfection, the cells were placed in fresh low-serum medium [0.05% (v/v) FCS] and incubated for a further 24 h prior to experimentation. For co-transfection exper-

Abbreviations used: BIM-I, bisindolylmaleimide I; FCS, foetal calf serum; GFP, green fluorescent protein; HA, haemagglutinin; MEF, mouse embryonic fibroblast; PKC, protein kinase C; PP1, protein phosphatase 1; PP2A, protein phosphatase 2A; T505A (etc.), Thr⁵⁰⁵ → Ala mutant.

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iments, a 1:2 ratio of myc-PKC δ and haemagglutinin (HA)-ubiquitin (in pMT 123) plasmids was used.

Plasmid constructs

The rat PKC δ gene sequence was introduced into a pEGFPC3 vector between *Xho*I and *Kpn*I sites. For the construction of GFP-PKC δ , PKC δ was amplified by PCR from the pEflink vector using primers to introduce a *Xho*I restriction site 5' of the open reading frame and a *Kpn*I restriction site 3' of the open reading frame. The sequences flanking the PKC δ gene in the pEGFP C3 vector are as follows: 5'-GCCTCGAGATGGCACCGTTCTGCGC-3' (*Xho*I site) and 5'-CAATTCCTGGAA-GAATAGGGTACCCCC-3' (*Kpn*I site). Phosphorylation-site mutants were generated at the T-loop Thr⁵⁰⁵ site [Thr⁵⁰⁵ → Ala (T505A)], the autophosphorylation Ser⁶⁴³ site [Ser⁶⁴³ → Ala (S643A)] and the hydrophobic Ser⁶⁶² site [Ser⁶⁶² → Ala (S662A)]. Synthetic DNA oligonucleotide primers containing the desired mutations were designed, and site-directed mutagenesis was performed as described in the Quickchange[®] Mutagenesis kit (Stratagene). The PKC δ T505A mutation was made using the oligonucleotide 5'-GGAGAACCGGGCCAGCGCTTTCTGCGGCACTCCTGACTACATCG-3' and its reverse complement. The S643A mutation was made using the oligonucleotide 5'-CC-TGAATGAGAAACCCAGCTAGCCTTCAGTGACAAG-AACC-3' and its reverse complement. The S662A mutation was generated using the following oligonucleotide 5'-CCAGA-CAGCCTTCAAGGGCTTCGCATTCGTGAACCCCAAAT-ATGAGC-3' and its reverse complement. Similarly, the kinase-dead mutant Lys⁸⁷⁶ → Met (K376M) was made using the oligonucleotide 5'-GGAAAGGTACTTTGCAATTATGTACCTG-AAGAAGG-3' and its reverse complement.

Antibodies

For Western-blot analysis, a mouse monoclonal anti-(PKC δ) antibody, raised against amino acids 114–289 spanning the cysteine-rich region of human PKC δ , was used (Transduction Laboratories). Rabbit polyclonal anti-phospho antibodies were used to detect phosphorylation of the endogenous PKC δ at the T-loop site Thr⁵⁰⁵ and the autophosphorylation site Ser⁶⁴³ (Cell Signalling, New England Biolabs). In experiments where phosphorylation was detected on the overexpressed protein, the rabbit polyclonal antibodies employed were raised against the following phospho-peptides synthesized at the Cancer Research UK Peptide Synthesis Laboratory: RAST(P)FCGT (for Thr⁵⁰⁵ phosphorylation); PQLS(P)FSDK (for Ser⁶⁴³ phosphorylation); and KGFS(P)FVNP (for Ser⁶⁶² phosphorylation) [where single-letter amino-acid notation has been used and (P) indicates phosphorylation]. Peptides were coupled to keyhole-limpet haemocyanin and rabbits were immunized as described previously [18]. These antibodies were used at 1:3500 dilution. For immunoprecipitation, the 9E10 monoclonal anti-myc antibody was used. The anti-HA antibody was a rabbit polyclonal (Y-11) from Santa Cruz Biotechnologies, and the goat anti-actin polyclonal antibody was obtained from Transduction Laboratories.

Western blotting

Whole-cell extracts were harvested in Laemmli buffer [19]. Samples were boiled to 95 °C and stored at –20 °C or in liquid nitrogen (when analysing for phosphorylation status of proteins) prior to SDS/PAGE. Proteins were separated on 10% (w/v) acrylamide gels [8% (w/v) gels shown in Figure 3] and transferred

on to PVDF membranes (Millipore) using a wet transferring system (Bio-Rad Laboratories). Membranes were blocked for 1 h at 20 °C in PBS containing 0.1% Tween 20 and 5% (w/v) skimmed milk powder. When probing for phosphorylation sites, skimmed milk powder was substituted by 3% (w/v) BSA [20]. Membranes were incubated with primary antibody; phosphorylation-specific antibodies were incubated in the presence of the cognate dephospho-peptide overnight at 4 °C [21]. The membranes were washed, incubated with secondary antibody conjugated to horseradish peroxidase and immunoreactivity was visualized using an enhanced chemiluminescence detection system (ECL[®]; Amersham Biosciences). For a semi-quantitative comparison of protein bands, multiple exposures were obtained and exposures of films within the linear range (defined by the intensity ratios of immunoreactive bands from individual blots) were scanned with an Adobe UMAX magic scan, and relative band intensities were calculated using NIH image software.

Immunoprecipitation

Following a rinse with ice-cold PBS, a 100-mm-diameter dish of cells were scraped into 500 μ l of lysis buffer [50 mM Tris/HCl (pH 7.5), 2 mM EDTA, 0.2 M NaCl, 2 mM dithiothreitol, 10 mM benzimidazole, 0.2 mM PMSF, 5 mM *N*-ethylmaleimide, 10 μ g/ml leupeptin, 100 μ g/ml aprotinin, 10 μ M microcystin LR and 1% (w/v) Nonidet P40 (chemicals were from Alexis and Sigma)]. *N*-Ethylmaleimide was added to inhibit the action of de-ubiquitinating enzymes. Samples were snap-frozen in liquid nitrogen and homogenized using a glass Dounce homogenizer. After preclearing insoluble material, the lysate was incubated with 10 μ g of antibody, followed by incubation with Protein G-Sepharose beads (Sigma). The Protein G-immune complex was carefully washed three times in lysis buffer, boiled in Laemmli buffer and the immunoprecipitated proteins were subjected to SDS/PAGE on an 8% (w/v) acrylamide gel.

RESULTS

Calyculin A treatment promotes PMA-induced PKC δ degradation

To investigate whether phosphorylation of endogenous PKC δ affects the down-regulation process in a cell-type where PKC δ degradation is under cell-cycle control (see below), NIH 3T3 cells were incubated with or without the protein phosphatase 1 (PP1)/protein phosphatase 2A (PP2A) protein phosphatase inhibitor calyculin A prior to PMA treatment (Figure 1A). Unexpectedly, it was observed that PMA-induced degradation of PKC δ was greatly enhanced in the presence of calyculin A. This effect was not an epitope-related one, since myc-tagged PKC δ protein was similarly affected by calyculin A (50% degradation of myc-PKC δ with 6 h of PMA treatment increased to 84 ± 3% for PMA + calyculin A; mean ± range, *n* = 2). Neither was the increased degradation an effect of calyculin A alone (PKC δ protein ranged from 105–184% of the control with calyculin A alone). To ensure that calyculin A was actually affecting the phosphorylation state of PKC δ , phospho-specific antibodies specific for the T-loop site (Thr⁵⁰⁵) and the autophosphorylation site (Ser⁶⁴³) were employed. It was seen that in the presence of PMA, calyculin A significantly increased the phosphorylation at both of these phosphorylation sites. PMA treatment in the absence of calyculin A also led to an increase in the phosphorylation at these sites, but this was much less substantial. It was notable that, in the presence of PMA and calyculin A, not only was the extent of PKC δ phosphorylation higher, but also

Figure 1 Degradation of PKC δ

(A) PMA-induced degradation of PKC δ is enhanced by calyculin A. NIH 3T3 cells were serum-starved for 24 h and pretreated with or without calyculin A (10 nM) for 30 min prior to treatment with PMA (TPA; 400 nM) for 30 min. Cells were harvested and extracts were subjected to SDS/PAGE. Upper panel: endogenous PKC δ protein and phosphorylation at Thr⁵⁰⁵ [(P)T505] and Ser⁶⁴³ [(P)S643] were detected by Western blotting. Lower panel: quantification of protein and phosphorylation state. Results are means $\pm$ S.D. ($n = 3$). (B) The PKC inhibitor BIM-I blocks PKC δ degradation. Serum-starved NIH 3T3 cells were pretreated with or without 10 μ M BIM-I prior to PMA treatment (1 h) as indicated. Cells were harvested and extracts were subjected to SDS/PAGE. Endogenous PKC δ protein and phosphorylation at Thr⁵⁰⁵ [(P)T505] and Ser⁶⁴³ [(P)S643] were detected by Western blotting. This is one of three independent experiments. (C) PMA-induced degradation of PKC δ occurs in both wild-type (PKC $\alpha^{+/+}$) and PKC $\alpha^{-/-}$ MEFs. Wild-type and PKC $\alpha^{-/-}$ MEFs were treated with or without PMA (TPA; 400 nM) for the times indicated. Cell extracts were analysed for PKC δ and PKC α expression by Western blotting. * indicates a non-specific immunoreactive band, and the open arrowhead indicates the lack of PKC α in the knockout cell line. This is one of two independent experiments. Molecular-mass markers (in kDa) are indicated on the right.

the degree of PKC δ degradation. This was not a function of calyculin A alone since, in the absence of PMA, calyculin A had no effect upon PKC δ protein levels (results not shown). This suggests that, in contrast with the situation for PKC α [22], it is a hyperphosphorylated form of PKC δ that is targeted for degradation.

Consistent with an apparent requirement for hyperphosphorylation of PKC δ , degradation was found to be inhibited by the ATP-binding site inhibitor of PKC [23], bisindolylmaleimide I (BIM-I) (Figure 1B). Treatment with BIM-I prevented the PMA-induced increase in phosphorylation of the Ser⁶⁴³ autophosphorylation site as well as that at the Thr⁵⁰⁵ site; whether this is direct or not (e.g. through PKC δ traffic; see [24]) has yet to be elucidated. BIM-I has been shown to inhibit PKC α degradation [22] and traffic [24]. Since down-regulation can operate in 'trans' [25], it was determined whether PKC α was involved in the process of PKC δ down-regulation through use of wild-type (PKC $\alpha^{+/+}$) and PKC $\alpha^{-/-}$ mouse embryonic fibroblasts (MEFs). PMA treatment induced PKC δ loss in both cell types (Figure 1C). This clearly distinguishes the behaviour of PKC δ from that of PKC α and is consistent with the notion that the effect of BIM-I on PKC δ degradation is through PKC δ itself (see below).

PKC δ protein levels decrease upon entry into S-phase of the cell cycle

The cell-cycle dependence of PKC δ degradation is an effect that is determined by G₁-associated degradation, followed by inhibition of degradation on progression through G₁- and into S-phase [10]. To explore whether this pattern of cell-cycle-dependent down-regulation of PKC δ was observed in NIH 3T3 cells, subconfluent cells (5×10^5 cells in 100-mm-diameter dishes) were serum-starved for 24 h, followed by stimulation with fresh medium containing 10% (v/v) FCS. Western-blot analysis showed that PKC δ protein levels declined during G₁ and as cells entered into S-phase of the cell cycle (Figure 2A), as monitored by propidium iodide staining and FACS analysis (Figure 2A, middle and bottom panels). The levels of PKC δ recovered following S-phase entry and increased above initial levels as cells started to re-accumulate in G₁.

This serum-induced degradation of PKC δ was further investigated to see whether, similar to the PMA-induced degradation, calyculin A stimulated further loss of the protein and if BIM-I would inhibit this loss. As demonstrated in Figure 2(B), calyculin A treatment increased PKC δ degradation, whereas inhi-

Figure 2 NIH 3T3 cell PKC δ is down-regulated upon entry into S-phase

(A) Subconfluent NIH 3T3 cells were serum-starved for 24 h and stimulated to re-enter the cell cycle by the addition of serum for the times indicated. PKC δ was immunoblotted as shown in the top panel (this is one of four similar experiments). Results from two experiments (mean $\pm$ range) are shown in the middle panel (corrected for the actin content of cells; see upper panel). In the bottom panel, cell-cycle progression, determined by propidium iodide staining and FACS analysis, is shown. This is one of three cell-cycle analyses. (B) Cells were serum starved for 24 h followed by the re-addition of serum for up to 6 h. Where indicated, cells were pretreated with BIM-1 (10 μ M) or calyculin A (10 nM) for 30 min. Levels of PKC δ were determined by Western blotting. This is one of four independent experiments. (C) The phosphorylation state of PKC δ at the Thr⁵⁰⁵ (T505) and Ser⁶⁴³ (S643) sites from control (0 h) cells or cells after treatment with serum $\pm$ calyculin A (6 h) was determined by Western blotting, and results (means $\pm$ S.E.M.) from seven experiments are shown.

bition of PKC kinase activity by BIM-1 inhibited degradation of PKC δ (0.057 ± 0.06 serum alone; 0.080 ± 0.09 serum + BIM-1; means $\pm$ range, $n = 2$). Phosphorylation of the Thr⁵⁰⁵ and Ser⁶⁴³ sites was monitored in serum-stimulated cells (Figure 2C). Serum had minimal effect upon these sites, whereas serum + calyculin A induced a small, but consistent, increase in phosphorylation of both sites. Although the effects of serum $\pm$ calyculin A were modest with respect to Thr⁵⁰⁵ and Ser⁶⁴³ phosphorylation, the synergistic effect of calyculin A on this process indicates that for serum, as for PMA, the degradation of PKC δ occurs through a hyperphosphorylated form; whether this requires or is coincident with phosphorylation at these sites is detailed below.

Ubiquitination of PKC δ in PMA-treated NIH 3T3 cells

In order to verify that PKC δ becomes degraded via the ubiquitin/proteasome pathway, we attempted to identify ubiquitinated

species of the protein. To do this, myc-tagged PKC δ and HA-tagged ubiquitin were overexpressed (5–10-fold) in NIH 3T3 cells. Cells were treated with or without PMA and in the presence or absence of MG 132, a peptide aldehyde inhibitor of the 26 S proteasome [26]. Myc-PKC δ was immunoprecipitated from cells and PKC δ was detected predominantly as the 78 kDa form (Figure 3). Only from cells treated with both PMA and MG 132 was there a significant accumulation of high-molecular-mass forms of ubiquitinated PKC δ detected. Ubiquitinated PKC δ was also detected with both anti-ubiquitin (results not shown) and anti-HA antibodies (Figure 3, lower panel).

Thr⁵⁰⁵ phosphorylation is critical for efficient down-regulation of PKC δ

To determine which sites on PKC δ are important for degradation, GFP-tagged mutants were constructed to analyse the role of

Figure 3 PMA induces ubiquitination of PKC δ in NIH 3T3 cells

Myc-tagged PKC δ and HA-tagged ubiquitin (HA-Ub) were transiently overexpressed in NIH 3T3 cells. Cells were serum starved for 24 h and pretreated with or without MG 132 (25 μ M) for 30 min prior to stimulation with PMA (TPA; 400 nM) for 1 h. Myc-tagged PKC δ was immunoprecipitated using the 9E10 anti-myc antibody and separated by SDS/PAGE on an 8% (w/v) gel. PKC δ and association of HA-ubiquitin were detected by Western blotting. The arrows indicate slow migrating forms of PKC δ . Molecular-mass markers (in kDa) are shown on the left. This is one of three independent experiments.

individual phosphorylations of PKC δ . The behaviour of the wild-type tagged PKC δ and the mutants PKC δ T505A and PKC δ S662A were examined by Western blotting. Analysis of the alanine mutant of the Ser⁶⁴³ autophosphorylation site could not be conducted, since the expressed mutant protein was insoluble. Transiently transfected NIH 3T3 cells were treated with PMA for up to 6 h and the protein level and phosphorylation status of the PKC δ mutants were analysed (Figure 4). The T505A mutant protein did not degrade as rapidly as the wild-type PKC δ in response to PMA (Figure 4A). Phosphorylation was detected at both the Ser⁶⁴³ (autophosphorylation site) and Ser⁶⁶² (hydrophobic site) in unstimulated cells and phosphorylation rapidly diminished at these sites upon PMA treatment. Loss of phosphorylation at Ser⁶⁶² and Ser⁶⁴³ sites was more rapid than that seen for the wild-type protein. As expected for the T505A mutant, there was no immunoreactivity with the Thr⁵⁰⁵ phospho-specific antiserum.

Degradation of the S662A mutant in response to PMA occurred at a similar rate to the wild-type protein (Figure 4A). This correlated with a similar pattern of loss of the phosphorylated protein which, unlike the T505A mutant, was not rapidly dephosphorylated in response to PMA. Similarly, no immunoreaction was observed for the mutated site.

Since PKC δ expression can influence the cell-cycle progression of cells (for example, see [8,27,28]), the altered pattern of degradation for the T505A mutant might reside in changes to cellular behaviour independent of the protein itself. To address this, the mutant protein was co-expressed with the wild-type protein and their degradation rates were compared (Figure 4B). It is evident that the wild-type protein (myc-tagged) is more efficiently down-regulated than the T505A mutant (GFP-tagged) as quantified in Figure 4(C). To assess whether the altered behaviour of the T505A mutant was related to the degradation process characterized, the effect of BIM-I was determined. Although the degree of degradation was much reduced for the T505A mutant, it was evident that inhibition by BIM-I further

reduced degradation (Figure 4D) as for the wild-type protein (see above). Furthermore, in the absence of Thr⁵⁰⁵ phosphorylation there was no increase in degradation in response to calyculin A (Figure 4D).

Finally, to assess directly the intrinsic requirement for catalytic activity, the response of the kinase-dead K376M mutant of PKC δ was monitored. This inactive mutant was not degraded in response to PMA (Figure 4E). However, little or no phosphorylation at the three phosphorylation sites (Thr⁵⁰⁵, Ser⁶⁴³ and Ser⁶⁶²) was observed in the PKC δ K376M mutant under basal conditions and, furthermore, phosphorylation was not induced significantly in cells treated with calyculin A (results not shown), eliminating the possibility of a high turnover of phosphorylation.

DISCUSSION

The loss of function of PKC is a critical element in signals emanating from growth factors and PKC-directed tumour promoters; however, the triggers that effect this desensitization remain poorly defined. The present study shows that, unlike the classical isoform PKC α , dephosphorylation of PKC δ is not essential for its degradation. For PKC α , PMA-induced degradation of the protein can be blocked using inhibitors of PP1/PP2A [22,29,30]. In contrast, we show that treatment of cells with calyculin A, an inhibitor of the protein phosphatases PP1 and PP2A, increased the serum-stimulated and PMA-induced degradation of PKC δ . Evidence from monitoring phosphorylation-site occupancy and studies with mutants indicates that phosphorylation in the activation-loop at Thr⁵⁰⁵ is essential for efficient down-regulation.

The effect of calyculin A on PKC δ degradation indicates that its phosphorylation is a regulatory element in both PMA- and serum-induced degradation. Evidence that this effect is directed at PKC δ itself is reflected in the studies with the mutant forms of the protein. In particular, it is shown that the T505A mutant is degraded more slowly than the wild-type protein. This correlates with the substantial increase in phosphorylation of the Thr⁵⁰⁵ site observed when PKC δ is activated in the presence of PMA + calyculin A. Prolonged inhibition of PP2A can induce commitment to apoptosis and, although PKC δ is a substrate for caspases [31], the increased degradation observed in the present study appears to operate independently of this pathway, since the T505A mutant of PKC δ is insensitive to the effects of calyculin A despite retaining its caspase cleavage site. The rather more modest effect of calyculin A + serum on the phosphorylation of Thr⁵⁰⁵ might indicate that it is not the occupation of this site that determines the degradation, but rather that it may be required for a secondary event. However, it is not possible to conclude that this is indeed the case, since it is not possible to determine whether the rate-limiting step in the degradation process is the same in the two situations; if, for example, contrary to the effect of PMA, serum-induced phosphorylation is slow and the degradation fast, the steady-state level of the hyperphosphorylated intermediate would not be high.

Interestingly, the Ser⁶⁶² hydrophobic site is not rate-limiting for the PMA-induced degradation of PKC δ . Analysis by Western blotting demonstrated that the degradation of the hydrophobic-site alanine mutant, S662A, occurred in a similar fashion to that of the wild-type protein. This is important in deciphering the role of the Thr⁵⁰⁵ phosphorylation, because in the T505A mutant the Ser⁶⁶² site is dephosphorylated more rapidly. Hence, although it might have been surmised that Thr⁵⁰⁵ phosphorylation controls the rate of degradation through Ser⁶⁶², the fact that the S662A mutant is degraded as the wild-type protein indicates that this is not the case. In this context it is notable that, in the S662A

Figure 4 Analysis of site-directed mutants of PKCδ

(A) NIH 3T3 cells were transiently transfected with either wild-type GFP-tagged PKCδ (GFP-PKCδ) or alanine mutants of the Thr⁵⁰⁵ (T505A) and Ser⁶⁶² (S662A) phosphorylation sites. Cells were stimulated with PMA (TPA; 400 nM) for up to 6 h, subsequent to serum starvation for 24 h, and then harvested in Laemmli buffer. Western-blot analyses were performed for PKCδ protein expression and phosphorylation at Thr⁵⁰⁵ [(P)T505], Ser⁶⁴³ [(P)S643] and Ser⁶⁶² [(P)S662]. This is one of four similar experiments. (B) GFP-tagged T505A PKCδ (GFP-T505A) and myc-tagged wild-type PKCδ were co-expressed in NIH 3T3 cells and treated as described above. The proteins were detected with a PKCδ antiserum. This is one of two (2, 4 h) or three (0, 6 h) experiments. (C) Quantification of the relative rates of GFP-tagged T505A PKCδ and myc-PKCδ following PMA treatment for the times indicated. Results are means ± range. (D) Cells overexpressing GFP-tagged PKCδ T505A were serum-starved as above and pretreated with BIM-I (BIM; 10 μM) or calyculin A (Cal; 10 nM) as indicated for 30 min prior to PMA stimulation (TPA; 400 nM). Levels of PKCδ were determined by Western blotting. (E) The GFP-tagged kinase-dead mutant of PKCδ, K376M, was overexpressed in cells and treated and analysed as described in (A).

mutant, the Thr⁵⁰⁵ site is not dephosphorylated any more rapidly than the wild-type protein. This behaviour is distinct from that displayed by PKCα, where phosphorylation at each of the conserved sites has a 'protective' effect on dephosphorylation [20,32]. The implication is that either the kinase domain conformation of PKCδ is significantly different from that of PKCα or that there are other interacting factors that determine this behaviour.

The inhibition of degradation by the broad-specificity PKC inhibitor BIM-I is indicative of a requirement for PKC catalytic activity. Evidence that this is not directed through PKCα is provided by the observation that PKCδ degradation in PKCα^{-/-} MEFs is normal. The behaviour of the kinase-inactive K376M mutant of PKCδ suggests that degradation requires intrinsic activity. However, this mutant protein is poorly phosphorylated

at the conserved kinase domain sites, including the critical Thr⁵⁰⁵ site, and hence it remains possible that either activity or phosphorylation directs degradation. Similarly, the requirement for the phosphorylation of Thr⁵⁰⁵ could reflect either a conformational/recognition device or a requirement for catalytic activity [21]; at present it is not possible to distinguish between these mechanisms.

Under other cellular conditions, the degradation of PKCδ may occur in a similar fashion to PKCα, where the dephosphorylation of PKCδ precedes its degradation. However, in the present study, under conditions of degradation that can be regulated by cell-cycle progression, blockade of dephosphorylation promotes degradation. This pattern of response is similar to that described for other proteins, such as the proto-oncogene c-Myb and the

cell-cycle-regulated serine/threonine kinase Aurora2, where phosphorylation leads to their instability and degradation [33,34]. Several studies have suggested PKC δ as an inhibitor of cell-cycle progression. In support of this contention, we observed that overexpression of PKC δ delayed entry into S-phase in NIH 3T3 fibroblasts (results not shown) and that endogenous PKC δ levels decreased upon entry into S-phase, and subsequently recovered. The physiological down-regulation of PKC δ appears to be under cell-cycle control and the results indicate that activated PKC δ is recognized for degradation during G₀/G₁ for cell-cycle progression. As described previously for PMA treatment of 3Y1 cells [14], degradation of PKC δ in NIH 3T3 cells correlated with the recovery of polyubiquitinated forms. Whether the phosphorylation of PKC δ permits recognition by the relevant E3 component or whether activity is required for its physical delivery to this machinery remains to be determined. Ultimately, definition of the entire down-regulation pathway will provide the opportunity for intervention and hence elucidation of the role played by PKC δ in G₁ progression in normal and transformed cells.

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