Fused protein of δPKC activation loop and PDK1-interacting fragment (δAL-PIF) functions as a pseudosubstrate and an inhibitory molecule for PDK1 when expressed in cells

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To elucidate the role of 3-phosphoinositide-dependent protein kinase-1 (PDK1) in cellular signaling, we constructed and expressed a pseudosubstrate of PDK1, designated as δAL-PIF, and characterized its properties in cultured cells. δAL-PIF consists of two fused proteins of the protein kinase Cδ (δPKC) activation loop (δAL) and PDK1-interacting fragment (PIF). The phosphorylation of δAL-PIF was detected with anti-δPKC phospho-Thr⁵₀₅-specific antibody and was increased in proportion to the expression level of co-expressed GST-PDK1, indicating that it acts as a pseudosubstrate of PDK1. In cells expressing δAL-PIF, basal phosphorylation level at the activation loop of PKBα, δPKC and γPKC was reduced, compared with that in control cells, suggesting that δAL-PIF functions as an inhibitory molecule for PDK1. δAL-PIF affected the stability, translocation and endogenous activity of PKCs. These effects of δAL-PIF on γPKC properties were confirmed by investigation using conditioned PDK1 knockout cells. Furthermore, apoptosis frequently occurred in cells expressing δAL-PIF for 3 days. These findings revealed that δAL-PIF served as an effective pseudosubstrate and an inhibitory molecule for PDK1, suggesting that this molecule can be used as a tool for investigating PDK-mediated cellular functions as well as being applicable for anti-cancer therapy.

Introduction

Members of the AGC (cAMP-dependent, cGMP-dependent and protein kinase C) serine/threonine kinase family are important for various signal transduction pathways and regulate many cellular functions, including growth, differentiation and apoptosis. The activities of AGC kinases are regulated by phosphorylation at the activation loop, which commonly exists in the kinase domain of these kinases. 3-Phosphoinositide-dependent protein kinase-1 (PDK1) is an enzyme that phosphorylates the activation loop of many AGC kinases and regulates their activities (Belham et al. 1999; Toker & Newton 2000). PDK1 was first identified as the kinase that phosphorylates the threonine residue at the activation loop (Thr³⁰₈) of protein kinase βα (PKBα) in a phosphatidylinositol 3,4,5-triphosphate (PIP₃)-dependent manner, leading to the activation of PKBα (Alessi et al. 1997b; Stephens et al. 1998). PIP₃ is produced by the phosphoinositide 3-kinase (PI3K), which is activated by stimulation with growth factors. Therefore, PDK1 is a molecular mediator that links stimulations of growth factors with PKB/Akt activation. Later studies revealed that PDK1 also phosphorylates other AGC kinases, including protein kinase C (PKC) (Le Good et al. 1998), p70 ribosomal S6 protein kinase (S6K) (Pullen et al. 1998), p90 ribosomal S6 kinase (RSK) (Jensen et al. 1999) and serum and glucocorticoid-induced kinase (SGK) (Kobayashi & Cohen 1999). The essential role of PDK1 in activating AGC kinases was further confirmed by studies

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using mouse embryonic stem (ES) cells lacking PDK1 (Balandran et al. 2000; Williams et al. 2000). Furthermore, Lawlor et al. (2002) demonstrated that mice genetically lacking PDK1 were embryonic lethal, indicating that PDK1 is essential for mouse embryonic development. Additional studies using conditional PDK1 knockout mice have revealed that PDK1 is involved in viability and tolerance to hypoxia of cardiomyocytes (Mora et al. 2003), regulation of glyconeogenesis in the liver (Mora et al. 2005), T cell development in the thymus (Hinton et al. 2004) and glucose uptake in adipocytes (Sakaue et al. 2003). These findings suggest that PDK1 is indispensable for various cellular functions probably via its regulation of AGC kinases.

Among the various PDK1-mediated signaling pathways, the PI3K-PDK1-PKB/Akt pathway is the most intensively investigated. This pathway is activated by various growth factors and is known to regulate various cellular functions such as glucose uptake, glyconeogenesis, protein synthesis, transcription and cell survival (Alessi 2001). Aberrant regulation of PI3K-PDK1-PKB pathway is thought to be involved in oncogenesis (Fresno Vara et al. 2004; Osaki et al. 2004). Indeed, Kim et al. (2003) have recently demonstrated that RET/PTC (rearranged in transformation/papillary thyroid carcinomas) tyrosine kinase, a product from gene rearrangement frequently observed in thyroid cancers, activated PDK1 by directly phosphorylating its tyrosine residue in a PI3K-independent manner. Therefore, PDK1 has been focused on as a target for cancer therapy (Fresno Vara et al. 2004; Osaki et al. 2004).

In contrast to the PI3K-PDK1-PKB pathway, the role of PDK1 in PKC regulation has not been fully elucidated yet. The phosphorylation of PKC activation loop constitutively occurs in a PIP2-independent manner (Sonnenburg et al. 2001), which is different from the phosphorylation of PKB by PDK1. Furthermore, PDK1-induced phosphorylation at the activation loop does not directly activate PKC but causes the autophosphorylation of two serine/threonine residues (turn and hydrophobic motifs) by PKC and makes PKC catalytically competent (Behn-Krappa & Newton 1999). This catalytically competent PKC is fully activated by a phorbol ester or Gα protein-coupled receptor stimulation. Previous live imaging studies using green fluorescent protein (GFP)-tagged PKC (PKC-GFP) demonstrated that PKCs are translocated to several cellular organelles in a subtype- and stimulation-specific manner when activated by various stimulations (Sakai et al. 1997; Shirai et al. 1998, 2000). Thereafter, PKCs recognize and phosphorylate their target substrates and cause subsequent cellular responses (PKC targeting) (Olmori et al. 1998, 2000). These findings suggest that the isoform- and stimulation-specific translocation of PKC provides the molecular basis underlying the multiplicity of PKC functions. However, little is known about the role of PDK1 in PKC targeting.

To investigate how PDK1 regulates AGC kinases, including PKC and PKB/Akt, we attempted to determine the properties of these kinases under the condition in which PDK1 function was attenuated. For this purpose, we constructed a pseudosubstrate for PDK1 and over-expressed it in cultured cells as an inhibitory molecule of PDK1. In this report, we present results showing that the pseudosubstrate of PDK1 (δAL-PIF) actually inhibited PDK1 activity, affected the stability, kinase activity and targeting mechanism of γPKC and δPKC and induced apoptosis. We also discuss the experimental benefits and therapeutic utility of this pseudosubstrate.

Results

Confirmation of δAL-PIF as a pseudosubstrate for PDK1

For constructing a pseudosubstrate for PDK1, we considered that the peptide around the activation loop of PKC would be an artificial substrate for PDK1. Furthermore, Biondi et al. (2000) demonstrated that a PDK1-interacting fragment (PIF), which was derived from the carboxyl terminus of PKC-related kinase-2, interacted with PDK1 with high affinity. In addition, the PDK1-induced phosphorylation of the peptide around the activation loop of PKBα (T308tide), a PKD1 substrate, was increased by the attachment of PIF to T308tide. Therefore, in the present study, we constructed δAL-PIF, consisting of two pairs of the peptide around the δPKC activation loop (δAL) and PIF in tandem, as a pseudosubstrate for PDK1 (Fig. 1A).

To confirm that δAL-PIF functions as a pseudosubstrate of PDK1, we examined the expression and phosphorylation state of δAL-PIF-GFP in COS-7 cells by immunoblotting. As shown in Fig. 2, δAL-PIF-GFP was recognized as a band with a predicted size of 40 kDa by anti-GFP antibody. This protein was also detected by an antibody against phosphorylated Thr505 of δPKC. The phosphorylation of δAL-PIF-GFP was increased in proportion to the expression level of co-expressed GST-PDK1, while the expression level of δAL-PIF-GFP was not affected (Fig. 2). These results suggest that δAL-PIF was phosphorylated by PDK1 and could function as a pseudosubstrate of PDK1.

Influence of δAL-PIF on phosphorylation of the PKB activation loop

Next, we examined whether δAL-PIF-GFP overexpression affects PDK1-regulated AGC kinases including
PKBα or PKC family members. Since it is known that PDK1 enhances phosphorylation of the PKBα activation loop at Thr<sup>308</sup> after insulin-like growth factor-1 (IGF-1) stimulation (Williams et al. 2000), COS-7 cells expressing PKBα-GFP plus GFP (control) or PKBα-GFP plus δAL-PIF-GFP were treated with 50 ng/mL IGF-1 for 15 min, and amounts of expressed PKBα-GFP and phosphorylated PKBα-GFP at Thr<sup>308</sup> was examined by immunoblotting with anti-GFP and anti-PKBα phosphor-Thr<sup>308</sup> antibodies, respectively (Fig. 3). Phosphorylation level at Thr<sup>308</sup> of PKBα-GFP (Fig. 3C) was evaluated by the ratio of the amount of phospho-PKBα-GFP to that of expressed PKBα-GFP. As shown in Fig. 3C, in cells expressing PKBα-GFP, the phosphorylation level of Thr<sup>308</sup> was significantly increased by 15-min treatment with 50 ng/mL IGF-1 (145.3 ± 13.4% of that in non-treated cells). In cells expressing PKBα-GFP plus δAL-PIF-GFP, the IGF-1-induced enhancement of phosphorylation was not affected by δAL-PIF-GFP (145.8 ± 15.7% of that in non-treated cells) (Fig. 3C). However, in cells without IGF-1 treatment, the basal phosphorylation level of Thr<sup>308</sup> was significantly decreased by δAL-PIF-GFP (68.2 ± 8.4% of that in cells expressing PKBα-GFP plus GFP). In all experimental conditions, neither IGF-1 treatment nor expression of δAL-PIF-GFP affected the expression level of PKBα-GFP (Fig. 3A,B). These results indicate that δAL-PIF reduced basal phosphorylation level of PKBα-GFP but did not affect either the IGF-1-induced phosphorylation or the expression level of PKBα-GFP.

**Influence of δAL-PIF on the properties of γPKC and δPKC**

We examined whether δAL-PIF affects the expression and phosphorylation levels of two subtypes of PKC, γPKC and δPKC. As shown in Fig. 4A,B, as the amount of co-expressed δAL-PIF-GFP was increasing, the expression level of γPKC-GFP tended to be decreased, although the statistical significance was not obtained. In contrast, δAL-PIF-GFP strongly and significantly reduced...
the phosphorylation at Thr$^{505}$ of δPKC-GFP (Fig. 4A,B). When 4 and 8 μg of δAL-PIF-GFP plasmid was transfected, the phosphorylation level (the ratio of phosphorylated to expressed PKC-GFP) was reduced to 39.6 ± 3.2% and 32.2 ± 6.1% of that in the absence of δAL-PIF-GFP, respectively (Fig. 4C). In the case of γPKC-GFP, however, δAL-PIF-GFP strongly decreased the amount of expressed γPKC-GFP (Fig. 5A). When 4 and 8 μg δAL-PIF-GFP plasmid was transfected, the amount was significantly decreased by 21.4 ± 1.9% and 60.1 ± 3.7%, respectively (Fig. 5B). Likewise, the phosphorylation level of γPKC-GFP at Thr$^{514}$ was decreased by 36.0 ± 3.3% and 58.0 ± 9.6%, respectively (Fig. 5C). These results suggest that δAL-PIF strongly inhibited phosphorylation of the γPKC and δPKC activation loop, while it decreased the expression level of γPKC more prominently than that of δPKC.

Next, we explored whether δAL-PIF-GFP influences the function of endogenous PKCs. To address this issue, we focused on MARCKS (myristoylated alanine-rich C-kinase substrate), a major substrate of PKC (Blackhear 1993). MARCKS is myristoylated at Gly$^2$ near its amino terminus and is localized on the plasma membrane.

**Figure 2** δAL-PIF-GFP was phosphorylated by PDK1 in COS-7 cells. COS-7 cells were transfected with indicated amounts of plasmids and were harvested 2 days after transfection. Upper, middle and lower images show the results of immunoblotting with anti-GFP, anti-δPKC phosphor-Thr$^{505}$ and anti-PDK1 antibodies, respectively. Anti-GFP and anti-δPKC phosphor-Thr$^{505}$ antibodies detected δAL-PIF-GFP with a molecular weight of around 40 kDa. Anti-PDK1 antibody detected GST-PDK1 with a molecular weight of around 90 kDa. The extent of δAL-PIF-GFP phosphorylation is augmented in accordance with increasing amount of transfected GST-PDK1. The images are representative of three experiments.

**Figure 3** δAL-PIF-GFP inhibited basal phosphorylation but not IGF-1 (50 ng/mL)-induced phosphorylation of the PKB$\alpha$-GFP activation loop in COS-7 cells. COS-7 cells were transfected with PKB$\alpha$-GFP (10 μg) and GFP/δAL-PIF-GFP (10 μg). One day after the transfection, FBS was eliminated from the culture medium. After an additional one day of cultivation, cells were stimulated with IGF-1 (50 ng/mL) for 15 min at 37 °C, immediately followed by cell harvest. The expression of GFP-fused protein and the phosphorylation of PKB$\alpha$-GFP were detected by immunoblotting. (A) Representative immunoblotting images of six experiments with anti-GFP (left) and anti-PKB$\alpha$ phosphor-Thr$^{308}$ (right) antibodies. δAL-PIF-GFP was also detected with anti-PKB$\alpha$ phosphor-Thr$^{308}$ antibody (right). The immunoreactive bands around 75 kDa in left image would be the degradation product of δPKC-GFP. (B) Relative amount of expressed (left) and Thr$^{308}$-phosphorylated (right) PKB$\alpha$-GFP was quantified by the intensities of approximately 90-kDa-immunoreactive bands detected with anti-GFP antibody or anti-phosphor-Thr$^{308}$ antibody, respectively. (C) The phosphorylation level is indicated as the ratio of the amount of phosphorylated PKB$\alpha$-GFP to that of expressed PKB$\alpha$-GFP in each sample. Data are presented as the percentage of the value obtained from cells transfected with PKB$\alpha$-GFP and GFP without IGF-1 treatment (mean ± SEM of six experiments). *P < 0.05, **P < 0.01 vs. non-stimulated cells, †P < 0.05 (unpaired t-test).
**A**

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**注**
MARCKS is phosphorylated at multiple serine residues (Ser<sup>132</sup>, Ser<sup>136</sup> and Ser<sup>163</sup>, Fig. 1C) by PKC when the receptor-mediated stimulation translocates PKC to the plasma membrane. Replacement of Gly<sup>2</sup> with alanine (G2A mutation, Fig. 1C) results in a non-myristoylated MARCKS that is localized in the cytoplasm. We previously demonstrated that this non-myristoylated mutant MARCKS (MARCKS-G2A) was a PKC substrate in the cytoplasm (Ohmori et al. 2000), suggesting that the basal activity of PKC can be evaluated by the phosphorylation level of MARCKS-G2A. Therefore, we examined whether δAL-PIF affects the basal kinase activity of endogenous PKC in COS-7 cells through the phosphorylation level of co-expressed MARCKS-G2A-GFP. The phosphorylation level of MARCKS-G2A-GFP was greatly decreased by δAL-PIF-GFP (56.6 ± 3.9% of control), while the amount of expressed MARCKS-G2A-GFP was not significantly changed (Fig. 6), indicating that δAL-PIF inhibited the basal activity of endogenous PKC in COS-7 cells.

We have recently demonstrated that the retention period at the plasma membrane was prolonged in the case of translocation of mutant γ and δPKC-GFPs with an unphospho-activation loop (Seki et al. 2005). Since δAL-PIF inhibited the activation loop phosphorylation of γPKC and δPKC (Figs 4 and 5), we examined whether δAL-PIF affected the P2Y receptor-mediated translocation of γPKC-GFP and δPKC-GFP in δAL-PIF-expressing cells, δAL-PIF was fused with another fluorescent protein, DsRed2 (δAL-PIF-DsRed2). We confirmed that δAL-PIF-DsRed2 inhibited the phosphorylation at the activation loop of γPKC-GFP and δPKC-GFP in COS-7 cells, in a manner similar to that of δAL-PIF-GFP (data not shown). First, we examined the effects of δAL-PIF-DsRed2 on δPKC-GFP translocation in CHO cells. ATP, an agonist of P2Y receptor, was used for the induction of PKC translocation. As shown in Fig. 7A, in DsRed2-expressing control cells, ATP (1 mM) induced rapid translocation of δPKC-GFP from the cytoplasm to plasma membrane within 30 s, followed by re-translocation from the membrane to cytoplasm within 1 min after the stimulation. In δAL-PIF-DsRed2-expressing cells, although δPKC-GFP was rapidly translocated to the plasma membrane by the stimulation in a manner similar to that in the control cells, it was retained at the plasma membrane for a longer period and was re-translocated to the cytoplasm around 2 min after the stimulation (Fig. 7B). Similar results were obtained in case of γPKC-GFP (Fig. 8). γPKC-GFP in δAL-PIF-DsRed2-expressing cells was retained at the plasma membrane for more than 2 min after the stimulation (Fig. 8B). δAL-PIF-DsRed2 itself was not translocated by the same stimulation (Figs 7B and 8B). These results indicate that δAL-PIF prolonged the retention period of γPKC-GFP and δPKC-GFP at the plasma membrane after translocation from the cytoplasm.

**Effects of δAL-PIF on binding between PKC and PDK1**

It has been reported that PDK1 binds to PKC through the carboxyl terminus region of PKC (Gao et al. 2001). We next examined whether δAL-PIF influences the interaction between PKC and PDK1. GST-PDK1 was transiently co-expressed with γPKC-GFP or δPKC-GFP in COS-7 cells. δAL-PIF-GFP or GFP was additionally expressed in COS-7 cells expressing GST-PDK1 and PKC-GFP. Interaction between PKC-GFP and GST-PDK1 in cells expressing δAL-PIF-GFP was compared with that in cells expressing GFP by GST pull-down assay. In cells expressing GFP (control cells), γPKC-GFP and δPKC-GFP were precipitated with GST-PDK1 (Fig. 9, lanes 1 and 3). However, in cells expressing δAL-PIF-GFP, γPKC-GFP and δPKC-GFP were not detected in the precipitated sample (Fig. 9, lanes 2 and 4), although δAL-PIF-GFP did not affect the expression level of γPKC-GFP and δPKC-GFP (Fig. 9, lanes 5–8). Instead of PKC-GFP, δAL-PIF-GFP was precipitated with GST-PDK1 in these cells (Fig. 9, lanes 2 and 4). These results indicate that δAL-PIF competitively prevented the binding between PDK1 and PKC, resulting in reduction of phosphorylation at the PKC activation loop.

In contrast to the result in Fig. 5, the expression of γPKC-GFP was not affected by δAL-PIF-GFP (Fig. 9, lane 5).
Induction of apoptosis by δAL-PIF over-expression

In the present series of studies, we were aware that treatment with δAL-PIF for more than 2 days caused cell death. We examined whether the expression of δAL-PIF induced apoptosis in COS-7 cells by observing chromatin condensation. Three days after the transfection of δAL-PIF-GFP or GFP, cell nuclei were stained with Hoechst33342. Cells with condensed or fragmented nuclei were considered to be apoptotic cells (Fig. 10A, arrows). Most of the COS-7 cells transfected with GFP alone did not have apoptotic nuclei (Fig. 10A, upper), while condensed or fragmented nuclei were frequently observed in cells expressing δAL-PIF-GFP alone (Fig. 10A, middle). However, additional expression of GST-PDK1 decreased the frequency of apoptotic cells in cells expressing δAL-PIF-GFP (Fig. 10A, lower). We calculated the percentages of apoptotic cells in GFP fluorescing cells (Fig. 10B). In control GFP-expressing cells, 12.7 ± 3.1% of the cells were apoptotic and the extent of apoptosis was not affected by the coexpression of GST-PDK1 (12.1 ± 2.4%). In δAL-PIF-GFP-expressing cells, the percentage of apoptotic cells was significantly increased (40.8 ± 3.2%, P < 0.01) compared with the percentage of apoptotic cells expressing GFP alone. This effect of δAL-PIF-GFP was significantly reversed by the co-expression of GST-PDK1 (20.6 ± 1.8%, P < 0.01). These results suggest that δAL-PIF induced apoptosis via inhibiting the activity of PDK1 in COS-7 cells.

Confirmatory studies on δAL-PIF properties using PDK1 knockout cells

In this study, we found that δAL-PIF reduced the basal phosphorylation level of PKCδ, affected the stability, phosphorylation, kinase activity and receptor-mediated translocation of PKC and induced apoptosis. These findings strongly suggest that δAL-PIF exerted these effects by inhibiting the PDK1-mediated signaling. To further confirm that δAL-PIF prevents PDK1 functions, we examined whether the findings obtained in δAL-PIF-expressed cells were also observed in cells in which PDK1 was deficient. For that purpose, we used conditional PDK1 knockout cells. We established immortalized mouse embryo fibroblast (MEF) cells derived from PDK1lox/lox and PDK1+/+ (wild-type) mice by the 3T3 protocol (Todaro & Green 1963) and designated them F/F and WT MEF cells, respectively. To disrupt the PDK1 gene in F/F MEF cells, we introduced Cre recombinase into cells by using an adenoviral vector (Ad-Cre). Infection of Ad-Cre (MOI = 50) greatly decreased the expression level of endogenous PDK1 in F/F cells 4 days after infection, compared with that in cells infected with the control adenoviral vector, Ad-tTA (adenoviral vector to express tetracycline transactivator) (Fig. 11C, lower). In contrast, the amount of PDK1 in WT MEF cells was not affected by infection of Ad-Cre (Fig. 11B, lower). To examine whether PDK1 deficiency affects the stability of PKC, we selected γPKC-GFP whose expression level was affected by δAL-PIF, and expressed it into WT and F/F MEF cells. We infected F/F MEF cells with Ad-γPKC-GFP (adenoviral vector to express γPKC-GFP) on the day after the first infection with Ad-Cre or Ad-tTA (Fig. 11A). Three days after the infection of Ad-γPKC-GFP, the amount of expressed γPKC-GFP was decreased in F/F MEF cells infected with Ad-Cre compared with that in cells infected with Ad-tTA (Fig. 11C, upper). The expression level of γPKC-GFP in WT MEF cells infected with Ad-Cre was similar to that in WT MEF cells infected with Ad-tTA, suggesting that the result was not due to the difference in adenoviral vectors (Fig. 11B, upper).

To examine the receptor mediated-translocation of γPKC-GFP under the condition of PDK1 deficiency, we further established F/F MEF cells that stably express Cre recombinase and designated them as F/FCre MEF cells. As control cells, we generated WT MEF cells stably expressing Cre recombinase and designated them as WTCre.
Figure 6 δAL-PIF-GFP inhibited endogenous PKC activity. Effect of δAL-PIF-GFP on phosphorylation level of non-myristoylated MARCKS mutant (MARCKS-G2A), a substrate of PKC, was examined. MARCKS-G2A-GFP (10 µg) plus δAL-PIF-GFP (10 µg) or MARCKS-G2A-GFP (10 µg) plus an empty vector (10 µg) was transfected into COS-7 cells. After 3-day cultivation, the expression of GFP-fused proteins and the phosphorylation of MARCKS-G2A-GFP were detected by immunoblotting. (A) Representative immunoblotting images of four experiments with anti-GFP (left) and anti-phospho-MARCKS (right) antibodies. The immunoreactive bands around 60 kDa in right image were considered to be nonspecific band since endogenous MARCKS would be detected around 80 kDa. (B) Relative amount of expressed (left) and phosphorylated (right) MARCKS-G2A-GFP was quantified by the intensities of approximately 140-kDa-immunoreactive bands detected with anti-GFP antibody and anti-phospho-MARCKS antibody, respectively. (C) The phosphorylation level is indicated as the ratio of phosphorylated to expressed MARCKS-G2A-GFP in each sample. Data are presented as the percentage of the value obtained from cells transfected with MARCKS-G2A-GFP alone (mean ± SEM of five experiments). *P < 0.05, ***P < 0.001 vs. MARCKS-G2A-GFP alone (unpaired t-test).
MEF cells. Plasmids for γPKC-GFP were co-transfected with mGluR1 into WTCre and F/FCre MEF cells by lipofection. After 2 days of cultivation, glutamate (1 mM)-elicited γPKC-GFP translocation was observed using a confocal laser scanning microscope. In control WTCre cells, γPKC-GFP was rapidly translocated from the cytoplasm to plasma membrane within 30 s after the stimulation and returned to the cytoplasm within 2 min (Fig. 12, upper images). In F/FCre cells, γPKC-GFP was also rapidly translocated to the plasma membrane, while it was retained at the plasma membrane for a longer period than that when γPKC-GFP was expressed in control cells (Fig. 12, lower images). These phenomena observed in PDK1 knockout cells were similar to those observed in δAL-PIF-expressed cells, suggesting that δAL-PIF affected PKC functions by inhibition of PDK1-mediated signaling.

Discussion

In the present study, we revealed that δAL-PIF functioned as a pseudosubstrate for PDK1 (Fig. 2) and reduced the basal phosphorylation level at the activation loop of co-expressed PKBα-GFP, γPKC-GFP and δPKC-GFP (Figs 3–5). On the other hand, δAL-PIF had no effect on the activation loop phosphorylation of PKBα induced by stimulation with IGF-1 (Fig. 3). These results suggest that PDK1 phosphorylates the activation loops of PKB and PKC in different manners, which might be reflected by the different dependency of PIP3 in phosphorylation processes of the two kinases by PDK1. Growth factor-induced phosphorylation and activation of PKB requires activation of PI3K and an increase in PIP3 (Alessi et al. 1997a), while the activation loop of PKC is constitutively phosphorylated by PDK1.

Figure 7 ATP (1 mM)-induced translocation of δPKC-GFP co-expressed with (A) DsRed2 and (B) δAL-PIF-DsRed2 in CHO cells. CHO cells were transfected with 0.5 µg of a plasmid encoding δPKC-GFP together with 2 µg of a plasmid encoding (A) DsRed2 or (B) δAL-PIF-DsRed2 and cultured for 2 days on a 3.5-cm-diameter glass-bottom dish. ATP was used as an agonist for endogenous P2Y receptor in CHO cells. Sequential fluorescence images of GFP- and DsRed2-fusion protein in response to 1 mM ATP were monitored for 5 min using a confocal laser microscope. Images before (pre) and 0.5, 1, 1.5, 2 and 3 min after ATP stimulation are shown. In DsRed2-expressed cells, δPKC-GFP completely returned to the cytoplasm 1 min after ATP treatment, while it was still retained at the plasma membrane in cells expressing δAL-PIF-DsRed2. Images are representative of at least 5 experiments. Bar = 10 µm.
Recent studies have revealed that a knock-in mutation disturbing the PIF-binding pocket of PDK1 did not affect IGF-1-induced phosphorylation of the PKB activation loop (Collins et al. 2003) but strongly inhibited phosphorylation of the PKC activation loop (McManus et al. 2004). On the other hand, the knock-in mutation of PDK1 that disturbed PIP3 binding resulted in elimination of IGF-1-induced phosphorylation of the PKB activation loop, while phosphorylation of the PKC activation loop was not affected (McManus et al. 2004).

This finding suggests that the PIF-binding pocket and PIP3-binding site of PDK1 are critical for phosphorylation of the PKC and PKB activation loops, respectively. Indeed, the present study demonstrated that δAL-PIF greatly disturbs the interaction between PKC and PDK1 (Fig. 9). δAL-PIF would preferably disturb PIF-dependent phosphorylation of the PKC activation loop, rather than PIP3-dependent phosphorylation of PKB.

In the present study, δAL-PIF dose-dependently decreased the amount of expressed γPKC-GFP (Fig. 4). However, it did not significantly alter the amount of δPKC-GFP, although it tended to be decreased (Fig. 5), suggesting that unphosphorylated δPKC-GFP is more stable than unphosphorylated γPKC-GFP. The instability of unphospho-γPKC-GFP was confirmed by experiments using PDK1 knockout MEF cells (Fig. 11). It has been reported that mutant γPKC, whose threonine residue at the activation loop (Thr514) was replaced with alanine, almost completely lacked kinase activity (Seki et al. 2005). In contrast, the kinase activity of δPKC was retained even if its threonine residue at the activation loop (Thr505) was replaced with alanine (Stempka et al. 1997; Seki et al. 2005). It is generally accepted that the
phosphorylation of turn and hydrophobic motifs around the carboxyl terminus is involved in the stability of kinase to high temperature, oxidation and phosphatase (Bornancin & Parker 1996; Edwards & Newton 1997). Since autophosphorylation of these two sites requires the activity of PKC itself, the difference between the stability of δAL-PIF-affected γPKC-GFP and that in δPKC-GFP might be derived from their remaining kinase activities.

To explore whether δAL-PIF affect the basal activities of endogenous PKCs, we used MARCKS-G2A, which is a non-myristoylated MARCKS and is localized in the cytoplasm, as a PKC substrate. We have previously reported that phosphorylation of MARCKS-G2A-GFP was independent of the stimulations which lead to PKC translocation and activation (Ohmori et al. 2000), indicating that phosphorylation of MARCKS-G2A represents the basal kinase activity of PKC. As shown in Fig. 6, the basal kinase activity of endogenous PKC in COS-7 cells was significantly decreased by δAL-PIF. This result suggests that δAL-PIF inhibits phosphorylation of the activation loop of endogenous PKC as well as co-expressed PKC-GFP and subsequently reduces PKC-mediated cellular functions.

Live imaging experiments revealed that δAL-PIF did not inhibit P2Y receptor-mediated translocation of γPKC-GFP and δPKC-GFP from the cytoplasm to plasma membrane that was triggered by stimulation with 1 mM ATP but significantly prolonged the retention period of the PKC-GFP at the plasma membrane following translocation (Figs 7 and 8). In addition, the translocation of γPKC-GFP and δPKC-GFP occurred more remarkably in δAL-PIF-DsRed2-coexpressed cells than in DsRed2-coexpressed cells. This is consistent with our recent finding that the mutant γPKC-GFP and δPKC-GFP with an unphospho-activation loop were retained at the plasma membrane for a longer period than the wild-type in the case of receptor-mediated translocation (Seki et al. 2005). Similar results were also obtained in PDK1 knockout MEF cells (Fig. 12). Taken together, the results of the present study suggest that δAL-PIF affects PKC targeting by its interfering actions on PDK1.

Based on the previous investigations (Sakai et al. 1997; Oancea & Meyer 1998), it is considered that PKC movement is driven by diffusion, but not by ATP-dependent motor protein. In other words, translocation of PKC is considered to be a phenomenon that freely moving PKC molecules in the cytoplasm are trapped at the plasma membrane where diacylglycerol (DG) are accumulated after the receptor activation. We have previously demonstrated that kinase-negative PKC-GFP retained at the plasma membrane for longer than wild-type, suggesting that PKC activation accelerates the DG degradation (Seki et al. 2005). As shown in the present study, δAL-PIF decreased PKC kinase activity by inhibiting the activation loop phosphorylation by PDK1. Attenuation of PKC kinase activity would prevent the DG degradation and subsequently cause the intense accumulation of DG at the plasma membrane, resulting in the remarkable
Figure 10 δAL-PIF-GFP functions as an apoptosis inducer in COS-7 cells. COS-7 cells were transfected with GFP alone (0.5 µg), δAL-PIF-GFP alone (0.5 µg) and δAL-PIF-GFP (0.5 µg) plus GST-PDK1 (2 µg). Three days after transfection, cells were stained with Hoechst 33342 (50 µg/mL). The fluorescence of GFP and Hoechst 33342 was observed using a confocal laser microscope. Apoptotic cells were evaluated by chromatin condensation and fragmentation of nuclei stained by Hoechst 33342. A, Representative GFP (left) and Hoechst 33342 (right) fluorescence images of cells transfected with GFP alone (upper), δAL-PIF-GFP alone (middle) and δAL-PIF-GFP plus GST-PDK1 (lower). Arrows in the middle images indicate apoptotic cells having condensed or fragmented nuclei. The images are representative of three experiments. Bar = 10 µm. B, Percentage of apoptotic cells in total GFP-fluorescing cells. Each value is the mean ± SEM of three experiments. δAL-PIF-GFP significantly increased the percentage of apoptotic cells, and this effect was reversed by co-expression of GST-PDK1. **P < 0.01 (unpaired t-test).
PKC translocation from cytoplasm to membrane and the prolonged retention of PKC at plasma membrane.

We found that δAL-PIF induced apoptosis in COS-7 cells 3 days after the transfection of δAL-PIF. Similar results were obtained in PC12 cells, a neuronal cell line (data not shown). Since this effect was significantly reversed by the co-expression of GST-PDK1 (Fig. 10), δAL-PIF induced apoptosis by inhibiting PDK1. As PKB has an anti-apoptotic effect and is involved in cell survival and proliferation (Downward 1998), these results might reflect the inhibitory effect of δAL-PIF on PKB. However, δAL-PIF inhibited basal phosphorylation of the PKBα activation loop by about 30% but did not inhibit IGF-1-induced phosphorylation (Fig. 3). On the other hand, δAL-PIF strongly inhibited the activation loop phosphorylation of PKC (Figs 4 and 5). Many studies have revealed that PKC activation suppresses the apoptosis and cell death (Ruvolo et al. 1998; Bronisz et al. 2002). Therefore, it is possible that apoptosis induced by δAL-PIF might result from reduction of PKC activity.

Since PDK1 regulates the activities of many AGC kinases, including PKB and PKC, PDK1 is thought to play a key role in cell growth, survival and proliferation (Vanhaesebroeck & Alessi 2000; Mora et al. 2004), leading to the idea that PDK1 is a target for cancer therapy (Fresno Varà et al. 2004; Osaki et al. 2004). Indeed, reagents that inhibit PDK1 would be useful candidates for therapy against cancer (Ballif et al. 2001; Arico et al. 2004).
2002; Sato et al. 2002); however, specific PDK1 inhibitors have not been found yet. In addition, it is possible that systemic application of PDK1 inhibitors causes generalised severe side-effects. Considering the fact that δAL-PIF is a strong apoptosis inducer, regional gene therapy using a virus vector carrying δAL-PIF would be effective for solid cancers.

In addition to its therapeutic utility, the present study demonstrated that δAL-PIF would be a convenient and beneficial tool to elucidate the roles of PDK1 in various types of cells and tissues. If δAL-PIF is applied for generating region-specific and inducible transgenic mice using a tetracycline-regulated system (Sakai et al. 2004), temporal and regional functions of PDK1 in vivo could be investigated.

In conclusion, we revealed that δAL-PIF served sufficiently as a pseudosubstrate and an inhibitory molecule for PDK1. This molecule would be an effective material for anti-cancer therapy as a potent inducer of apoptosis as well as a beneficial tool for investigating PDK-mediated cellular functions in a variety of tissues.

**Experimental procedures**

**Materials**

ATP was purchased from Research Biochemical International (Natick, MA, USA). Insulin-like growth factor 1 (IGF-1) was from Invitrogen (Carlsbad, CA, USA). Anti-GFP rabbit polyclonal antibody was from Molecular Probes (Leiden, Netherlands). Anti-phospho-PKB (Thr308) rabbit polyclonal antibody was from Upstate Cell Signaling Solutions (Charlottesville, VA, USA). Anti-phospho-PKCγ (Thr547) rabbit polyclonal antibody was from Biosource International (Camarillo, CA, USA). Anti-phospho-PKCδ (Thr506) rabbit polyclonal antibody was from Cell Signaling Technology (Beverly, MA, USA). Anti-phospho-MARCKS (myristoylated alanine-rich C-kinase substrate) rabbit polyclonal antibody was generated as previously described (Yamamoto et al. 1998). Anti-PDK1 mouse monoclonal antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG and anti-mouse IgG antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). Hoechst 33342 was from Sigma Chemical Co. (St Louis, MO, USA).

**Plasmid construction**

We constructed a plasmid encoding PDK1 pseudosubstrate, designated as δAL-PIF (Fig. 1A), which consists of two pairs of two fragments, δAL and PIF in tandem. δAL is a 20-residue peptide around the activation loop of δPKC (GENRASCGTPTDNYATPEIR), which contains a threonine residue (Thr160) phosphorylated by PDK1. PIF (PDK1-interacting fragment) is the carboxyl terminal 24 peptide of PKC-related kinase 2 (REPRILSEEQEMFRDFDY1ADWC), which interacts with PDK1 (Biondi et al. 2000). Double-strand cDNA of δAL-PIF was made by hybridizing sense and anti-sense oligonucleotides corresponding to δAL-PIF amino acids sequences. The sense and...
anti-sense oligonucleotides used were 5'-aatagggagagcgc-ggcagcagatcggggccagctgttctgcgtcggatcggagcagaga-agtggatttgcagatgctagttgccggtc-3' and 5'-agtctatccagcagacagttggtgctcctcccaccttccttctccgaaagggtttcctgcgtcggatcggagcagaga-agtggatttgcagatgctagttgccggtc-3', respectively. Hybridization of oligonucleotide was performed in the presence of T4 kinase (TOYOBO, Osaka, Japan) and then harvested by 500 µg of protein of each sample was subjected to 7.5% SDS-PAGE, and the separated proteins were electrophoretically transferred on to polyvinylidene difluoride (PVDF) filters (Millipore, Bedford, MA, USA). Nonspecific binding sites on PVDF filters were blocked by incubation with 5% skim milk in PBS-T (0.01 M phosphate-buffered saline containing 0.03% Triton X) for > 1 h at room temperature (RT). After being washed with PBS-T, the PVDF filters were incubated with anti-GFP (diluted 1: 2000), anti-PKBα phosphor-Thr505 (1 : 500), anti-γPKC phospho-Thr514 (1 : 1000), anti-δPKC phospho-Thr516 (1 : 1000) or anti-phospho-MARCKS (1 : 500) rabbit polyclonal antibodies or anti-PDK1 (1 : 2000) mouse monoclonal antibody for > 1 h at RT. After further washing, the filters were incubated with HRP-conjugated anti-rabbit IgG (1 : 10000) or anti-mouse IgG (1 : 10000) antibodies for > 30 min at RT. After three more washes, the immunoreactive bands were visualized with a chemiluminescence detection kit (ECL™ Western Blotting Detection Reagents, Amersham Biosciences, Little Chalfont, UK). The band densities were quantified with Fluor-S MultiImager (Bio-Rad Laboratories). The phosphorylation level of each protein was evaluated by the ratio of band intensities of phosphorylated protein per totally expressed protein detected by anti-GFP antibody.

Cell culture

COS-7 cells were purchased from Riken Cell Bank (Tsukuba, Japan). The CHO-K1 cell strain was a gift from Dr Nishijima (National Institute of Health, Tokyo, Japan). COS-7 and CHO-K1 cells were cultured in Dulbecco’s modified Eagle’s medium and Ham’s F12 medium (Sigma Chemical Co.), respectively, supplemented with 10% fetal bovine serum (FBS), 100 units/mL of penicillin and 100 µg/mL of streptomycin in a humidified atmosphere containing 5% CO2 at 37 °C.

Immunoblotting

Transient transfection of COS-7 cells was performed by electroporation. Plasmids (16–30 µg) were transfected into 6–105 cells using a Gene Pulser (975 µF, 220V; Bio-Rad Laboratories, Hercules, CA, USA). Transfected cells were cultured in 9-cm-diameter culture dishes.

For immunoblotting, transfected cells were cultured for 2 days and then harvested by 500 g centrifugation. The cell pellet was washed with 1 mL of homogenate buffer (250 mM sucrose, 10 mM EGTA, 2 mM EDTA and 50 mM Tris/HCl, pH 7.4) and resuspended in 300 µL of lysis buffer (1% Triton X-100, 200 µg/mL of leupeptin, 1 mM PMSE, 1 mM sodium orthovanadate, 1 mM NaF and 100 µM Calyculin A) and then sonicated (UR-20P, TOMY SEIKO, Tokyo, Japan). Twenty to 30 µg of protein from each sample was subjected to 7.5% SDS-PAGE, and the separated proteins were electrophoretically transferred on to polyvinylidene difluoride (PVDF) filters (Millipore, Bedford, MA, USA). Nonspecific binding sites on PVDF filters were blocked with 5% skim milk in PBS-T (0.01 M phosphate-buffered saline containing 0.03% Triton X) for > 1 h at room temperature (RT). After being washed with PBS-T, the PVDF filters were incubated with anti-GFP (diluted 1: 2000), anti-PKBα phosphor-Thr505 (1 : 500), anti-γPKC phospho-Thr514 (1 : 1000), anti-δPKC phospho-Thr516 (1 : 1000) or anti-phospho-MARCKS (1 : 500) rabbit polyclonal antibodies or anti-PDK1 (1 : 2000) mouse monoclonal antibody for > 1 h at RT. After further washing, the filters were incubated with HRP-conjugated anti-rabbit IgG (1 : 10000) or anti-mouse IgG (1 : 10000) antibodies for > 30 min at RT. After three more washes, the immunoreactive bands were visualized with a chemiluminescence detection kit (ECL™ Western Blotting Detection Reagents, Amersham Biosciences, Little Chalfont, UK). The band densities were quantified with Fluor-S MultiImager (Bio-Rad Laboratories). The phosphorylation level of each protein was evaluated by the ratio of band intensities of phosphorylated protein per totally expressed protein detected by anti-GFP antibody.

Observation of translocation

Plasmids (2.5 µg) were transfected into CHO cells (1–105 cells) by lipofection using Fugene™ transfection reagent (Roche Molecular Biochemicals, Indianapolis, IN, USA) according to the manufacturer’s directions. Transfected CHO cells were spread on to poly-D-lysine-coated glass-bottom culture dishes (MatTek Corp., Ashland, MA, USA) and cultured for 2 days. Before the observation, the culture medium was replaced with 0.9 mL of HEPES buffer (135 mM NaCl, 5.4 mM KCl, 1 mM MgCl2, 1.8 mM CaCl2, 5 mM HEPES and 10 mM glucose, pH 7.3). The fluorescence of GFP and DsRed2 was monitored with a confocal laser scanning fluorescent microscope (LSM510META, Carl Zeiss, Esslingen, Germany) at 488-nm argon laser excitation using a 505–530-nm band pass barrier filter and 542-nm HeNe laser excitation using a 560-nm-long pass barrier filter, respectively. Translocations of GFP or DsRed2-fused proteins were triggered by direct application of 0.1 mL of ATP solution at a 10-times higher concentration into HEPES buffer to obtain the appropriate final concentration. Images were recorded every
cells from wild-type and PDK1 lox/lox mice were isolated and expressing GST-PDK1. This study was supported by a Grant-in-Aid for the Takeda Science Foundation, the Uehara Memorial Foundation and the Japanese Smoking Research Association. This work was carried out with equipment at the Analysis Center of Life Science, Hiroshima University and the Research Center for Molecular Medicine, Faculty of Medicine, Hiroshima University.

**Detection of apoptotic cells by nuclear staining**

Various sets of plasmids encoding GFP, ΔΔL-PIF-GFP, GST-PDK1 or an empty vector (Fig. 10B) were transfected into 1 × 10^5 COS-7 cells by lipofection, followed by cultivation for 3 days. Cells were stained with 50 μg/mL Hoechst 33342 for 30 min and harvested using a cell scraper. The fluorescence of Hoechst 33342 was monitored with a confocal laser scanning fluorescent microscope at 364-nm ultraviolet laser excitation using a 385–470-nm band pass barrier filter. We considered that cells with condensed or fragmented nuclei were apoptotic (Fig. 10A). We counted the number of such cells in 50–60 GFP-positive cells and calculated the percentage of apoptotic cells.

**Studies using PDK1 knockout cells**

Conditional knockout mice using a Cre/loxP system were generated by Inoue et al. (2006). Mice homozygous for a floxed PDK1 gene in which exons 3 and 4 were flanked by LoxP sequences were developed (PDK1lox/lox). Mouse embryonic fibroblast (MEF) gene in which exons 3 and 4 were flanked by LoxP sequences were developed (PDK1lox/lox). Mouse embryonic fibroblast (MEF) were immortalized according to the 3T3 protocol (Todaro & Green, 1963) and were designated as WT MEF and F/F MEF cells, respectively. To disrupt the PDK1 gene, we generated F/F MEF cells that stably express Cre recombinase using a retroviral vector (F/FCre MEF cells). In F/FCre MEF cells, no expression of PDK1 was confirmed by immunoblotting (data not shown). We also developed WT MEF cells that stably express Cre recombinase as control cells (WT Cre MEF cells).

To determine whether the expression level of γPKC-GFP was affected by PDK1 knockout, WT MEF and F/F MEF cells were infected with adenoviral vectors encoding Cre recombinase (Ad-Cre). We used an adenoviral vector encoding a tetracycline transactivator (Ad-ΔTATA) as a control vector. One day after the infection of Ad-Cre or Ad-ΔTATA, the cells were infected with an adenoviral vector encoding γPKC-GFP (Ad-γPKC-GFP) (Fig. 11A). In all adenovirus vectors used, expression of the target gene was driven by a CMV promoter. All infections were conducted at 50 multiplicity of infection (MOI). After a further 3 days of cultivation, the expression of γPKC-GFP and PDK1 was examined by immunoblotting.

To determine whether the receptor-mediated translocation of γPKC-GFP was affected by PDK1 knockout, F/FCre MEF and WTCre MEF cells were transfected with two plasmids encoding γPKC-GFP (0.5 μg) and metabotropic glutamate receptor type 1 (mGluR1) (2 μg) by magnetofection using Fugene™6 and CombiMag (OZ Biosciences, Marseille, France). Two days after transfection, the translocation of γPKC-GFP induced by 1 mM glutamate stimulation was observed as described above.

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