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Doxorubicin-induced activation of protein kinase D1 through caspase-mediated proteolytic cleavage: identification of two cleavage sites by microsequencing

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Abstract

Recent studies have demonstrated the importance of protein kinase D (PKD) in cell proliferation and apoptosis. Here, we report that in vitro cleavage of recombinant PKD1 by caspase-3 generates two alternative active PKD fragments. N-terminal sequencing of these fragments revealed two distinct caspase-3 cleavage sites located between the acidic and pleckstrin homology (PH) domains of PKD1. Moreover, we present experimental evidence that PKD1 is an in vitro substrate for both initiator and effector caspases. During doxorubicin-induced apoptosis, a zVAD-sensitive caspase induces cleavage of PKD1 at two sites, generating fragments with the same molecular masses as those determined in vitro. The in vivo caspase-dependent generation of the PKD1 fragments correlates with PKD1 kinase activation. Our results indicate that doxorubicin-mediated apoptosis induces activation of PKD1 through a novel mechanism involving the caspase-mediated proteolysis.

Keywords: Protein phosphorylation; Signal transduction; Enzyme regulation; Apoptosis; Mass spectrometry; Chemotherapeutic drugs

1. Introduction

Protein Kinase D1 (PKD1; also referred to as Protein Kinase Cμ) belongs to a novel protein kinase family, consisting of three isoenzymes: PKD1, PKD2 and PKD3, also called PKCυ [1–4]. The PKD enzymes have structural and enzymological properties distinct from all PKC isoforms. Their N-terminal regulatory domain contains a pleckstrin homology (PH) domain and a cysteine-rich region that mediates phorbol ester/diacylglycerol binding. The PKD catalytic domain shows little similarity to that of PKCs and possesses a distinct substrate specificity [5]. For reviews, see Refs. [6,7].

Several signaling molecules regulate and activate PKD, and phosphorylation by an upstream kinase is an important event in the activation process. G-protein-coupled receptors and receptor tyrosine kinases that activate phospholipase C (PLC) enzymes, activate PKD via the PKC-mediated phosphorylation of two serine residues in the activation loop of the kinase domain [8,9]. Although the PLC–DAG–PKC pathway for PKD activation is particularly important, there are also alternative mechanisms of PKD activation. For

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example, Gβγ subunits can interact directly with PKD in vitro and activate the enzyme in vivo as well as in vitro in a PKC-independent manner [10]. Furthermore, genotoxic chemotherapeutics, such as ara-C or etoposide, can cause caspase-mediated processing of PKD and the release of part of its regulatory/inhibitory domain [11].

Many anticancer chemotherapeutics which act through interference with DNA structure or replication also elicit profound signaling events that are crucial for their mechanism of action [12–16]. One of these drugs, doxorubicin (also known as adriamycin), is a much used anthracycline antibiotic with a broad spectrum of anticancer activity. Doxorubicin-induced DNA damage has been reported to cause cell cycle arrest at the G2-M phase [17], induction of apoptosis through caspase-3 and Bax [18], p53-dependent cytochrome *c* release [19], the production of reactive oxygen species [20] and activation of NFκB [21].

Here, we studied whether PKD1 could be activated by caspases upon induction of apoptosis with doxorubicin, and we identified the sites of cleavage. Our results indicate that PKD1 is a caspase substrate and that cleavage leads to activation in vitro and in vivo. The caspase-mediated activation of PKD1 is due to the removal of part of the regulatory/inhibitory domain of the kinase, releasing two alternative fragments containing the catalytic domain linked to the PH domain of the enzyme.

2. Materials and methods

2.1. Materials

zDEVD-fmk and zVAD-fmk were from Bachem (Bubendorf, Switzerland); Doxorubicin was from ICN; PROTRAN nitrocellulose transfer membranes were from Schleicher and Schuell (Dassel, Germany); γ^{32}P-ATP was from Amersham Life Sciences (Amersham, UK). All other materials were from Sigma (St. Louis, MO, USA).

2.2. In vitro screening of PKD1 cleavage by different caspases

Recombinant GST-PKD1 and caspases were expressed and purified as described [22,23]. Purified GST-PKD1 (100 ng) was incubated in buffer containing 10 mM Hepes, pH 7.4, 220 mM mannitol, 68 mM sucrose, 2 mM NaCl, 2.5 mM KH_2PO_4, 0.5 mM EGTA, 2 mM $MgCl_2$, 5 mM pyruvate, 0.1 μg/ml leupeptin, 0.1 mM phenylmethanesulfonylfluoride hydrochloride and 10 mM dithiothreitol (buffer A) with different caspases for 2 h at 37 °C. The reactions were stopped by adding SDS-PAGE sample buffer (1% SDS, 10% glycerol, 50 mM dithiothreitol and 12 mM Tris–HCl pH 6.8). The samples were boiled for 5 min and analysed by SDS-PAGE in gels containing 10% (w/v) acrylamide, followed by Western blotting [9] with antibodies that recognize the C-terminus of PKD.

2.3. PKD1 activity measurement after caspase-3-dependent cleavage

Purified PKD1-GST (7 μg) was incubated in Buffer A containing 0.1 mM MgATP, 0.1 mg/ml bovine serum albumin with or without recombinant caspase-3 (1 μg) for up to 90 min at 37 °C. Aliquots were taken at different times, and PKD1 activity was measured with syntide-2 peptide as substrate [23] under the conditions described in the legends to the figures. One unit of PKD1 activity corresponds to the amount of enzyme catalysing the formation of 1 nmol of product/min under the assay conditions.

2.4. Determination of caspase-3 cleavage sites in GST-PKD1

Purified PKD1-GST (24 μg) was incubated in Buffer A containing recombinant caspase-3 (2 μg) at 37 °C for

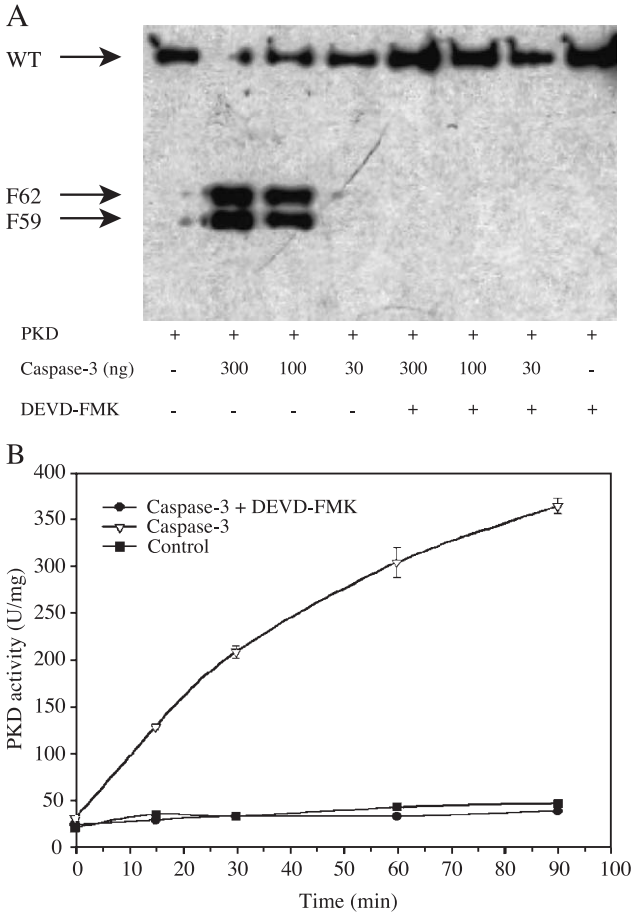

Fig. 1. PKD1 activation by caspase-3-dependent cleavage. (A) Purified GST-PKD1 was incubated for 1 h at 37 °C with the indicated amounts of recombinant caspase-3, with or without 100 μM DEVD-FMK. Reactions were stopped for analysis by SDS-PAGE and immunoblotting with an antibody recognizing the C-terminus of PKD1. (B) Purified GST-PKD1 was incubated with recombinant caspase-3, and aliquots were taken at the indicated times for PKD1 activity measurements at 30 °C in buffer containing 15 mM Tris–Cl, pH 8.0, 5 mM $MgCl_2$, 1 mg/ml bovine serum albumin, 500 μM [γ-^{32}P]MgATP (200 cpm/pmol) and 500 μM syntide-2 peptide (4). The values are the mean ± S.E. for three determinations.

up to 60 min. Aliquots were taken at different times and the reaction was stopped by adding SDS-PAGE sample buffer. Samples were analysed by SDS-PAGE in 10% acrylamide gels. Proteins were transferred to a Mini Pro-Blot PVDF membrane (Applied Biosystems) in 10 mM 3-cyclohexylamino-1-propanesulfonic acid (CAPS), pH 11, 10% (v/v) methanol. After electroblotting, the membrane was stained for 30 s in 0.1% (w/v) Amido Black. The protein bands of interest were cut and sequenced by Edman degradation.

2.5. Cell culture and preparation of extracts

A431 cells (ATCC CRL 1555) and A431 cells stably overexpressing PKD1 were grown as indicated before [9]. After incubation with doxorubicin (40 μM dissolved in H_2O) for the time periods indicated in the figures, cells were washed once with phosphate-buffered saline (PBS) and then lysed in buffer containing 50 mM Tris (pH 7.4), 1% Triton X-100, 1 mM aminoethyl-benzene sulfonyl fluoride (AEBSF), 2 mM EDTA, 2 mM EGTA, 1 mM DTT, 50 mM NaF and 200 μM microcystin. Lysates were centrifuged at 15,000 × *g* for 10 min, and the supernatants were either processed immediately or stored at − 20 °C.

2.6. Colorimetric MTT (tetrazolium) assay for determination of cell viability

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was dissolved in PBS at 5 mg/ml. Stock solution of MTT (100 μl) was added per ml medium in 24-well plates. Plates were incubated at 37 °C for 4 h. Acidic isopropanol (0.04 N HCl in isopropanol) was then added to all wells and mixed to dissolve the dark blue crystals. The plates were then read at a wavelength of 570 nm [24].

2.7. Immunoprecipitation and PKD activity measurements

Immunoprecipitation and kinase activity measurements of PKD1 were carried out as outlined before [9].

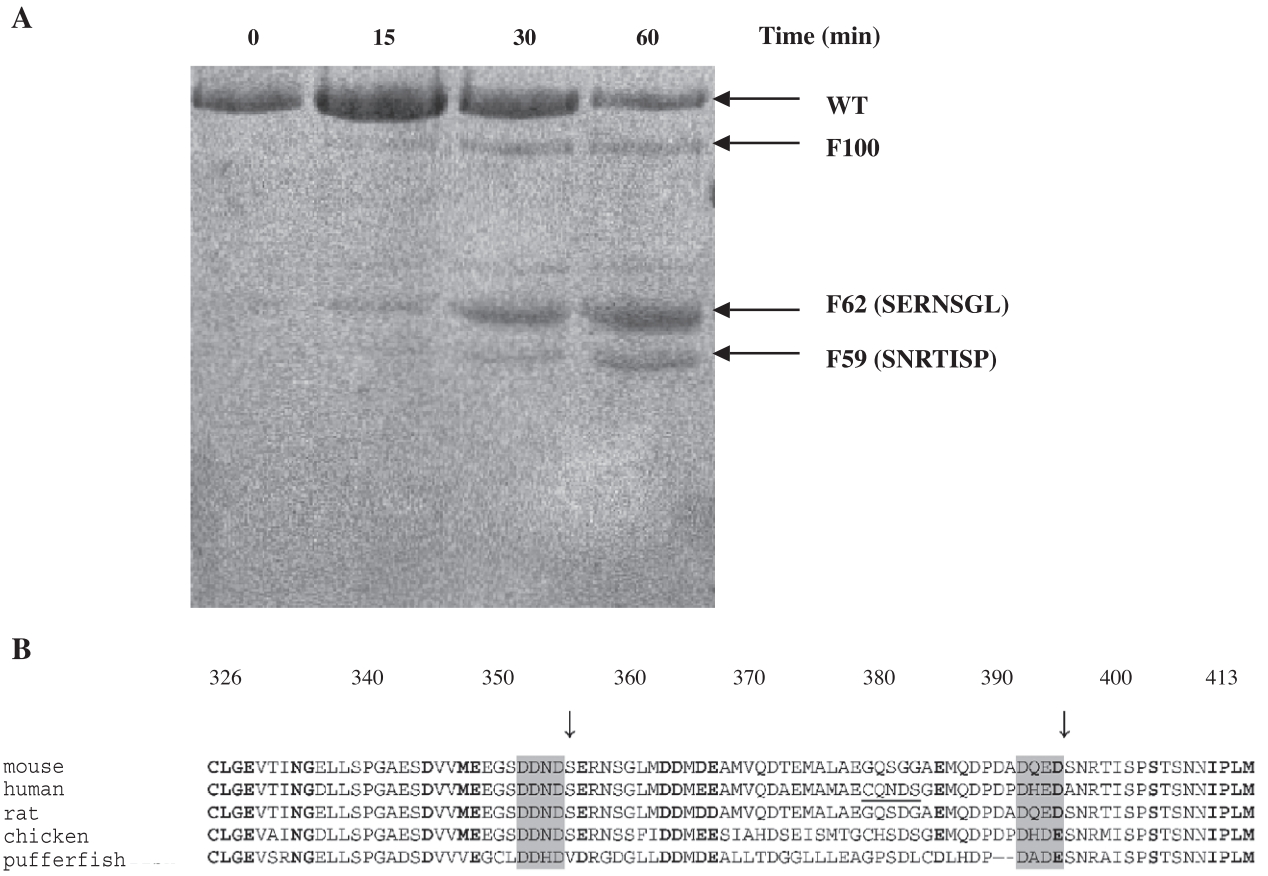

Fig. 2. Identification of caspase-3 cleavage sites in GST-PKD1. (A) Purified GST-PKD1 was incubated with recombinant caspase-3, and aliquots were taken at the indicated times. Proteins were separated by SDS-PAGE and transferred to a PVDF membrane for Amido Black staining. Each fragment was then subjected to Edman sequencing. Intact GST-PKD and the 59,000 Mr (F59), 62,000 Mr (F62) and 100,000 Mr (F100) fragments generated are indicated by ←. N-terminal sequencing results are shown for each fragment. (B) Alignment of potential caspase-3 cleavage sites in the PKD family. Conserved predicted caspase-3 sites are shaded in grey. Amino acids conserved among PKD1 in all species observed are in bold. The numbering above the alignment is based on mouse PKD1. The arrows indicate the location of the cleavage sites sequenced for mouse PKD1.

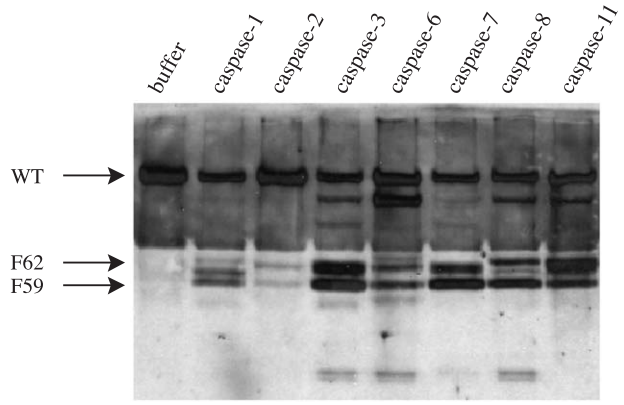

Fig. 3. Caspase-dependent cleavage of GST-PKD1. Purified GST-PKD1 was incubated with different recombinant caspases. Cleavage products were then analysed by SDS-PAGE/immunoblotting. Intact GST-PKD1 (WT) and the 100,000 Mr (F100), 59,000 Mr (F59) and 62,000 Mr (F62) fragments are indicated by →.

2.8. Western blot analysis

The protein samples were subjected to SDS-PAGE and transferred onto nitrocellulose transfer membrane (PROTRAN) according to standard procedures.

3. Results

3.1. Caspase-3 cleaves and activates PKD1 in vitro

PKCμ has been shown to be an in vitro substrate for caspase-3 [11], but the reported caspase-3 cleavage site ($CQND^{378}S$) in PKCμ, proposed from site-directed mutagenesis studies, is not conserved in PKD1 (see Fig. 2B). Recombinant PKD1-GST was incubated with recombinant caspase-3 for 1 h at 37 °C, separated by SDS-PAGE and analysed by Western blotting (Fig. 1A). Under these conditions, caspase-3 cleavage produced PKD1 fragments with relative masses of 59,000 and 62,000, respectively, that were recognized by the C-terminal anti-PKD antibody. Addition of DEVD-fmk, a specific caspase-3 inhibitor, completely abolished the processing of GST-PKD1 by caspase-3, indicating that cleavage was caspase-3-dependent. To determine whether the cleavage of PKD1 was associated with activation of the kinase, recombinant GST-PKD1 was incubated with caspase-3 and the kinase activity was measured (Fig. 1B). Cleavage of GST-PKD1 by caspase-3 was associated with a 14-fold increase in kinase activity, and this cleavage and activation was inhibited by DEVD-fmk (Fig. 1A,B).

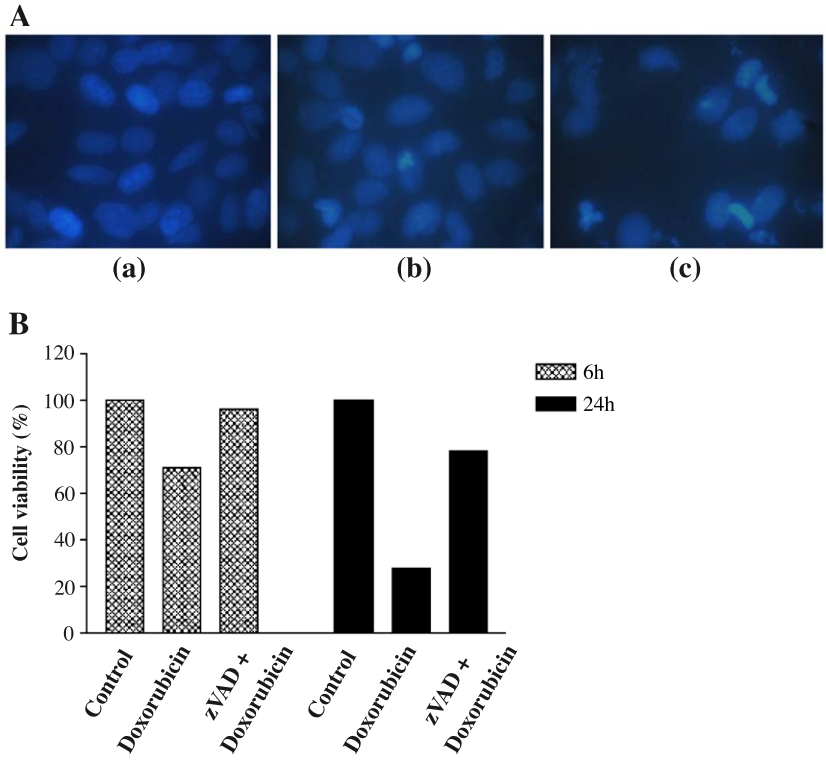

Fig. 4. Doxorubicin induces apoptotic cell death in A431-PKD1 cells. (A) A431-PKD1 cells were treated with or without 40 μM doxorubicin for 6 (b) or 24 (c) h. After methanol fixation, cells were incubated with 3 μM Hoechst 33258 dye. Nuclear staining was examined with a fluorescence microscope. (B) A431-PKD1 cells were treated with or without 40 μM doxorubicin for 6 or 24 h. Cell numbers were evaluated by MTT test. The results are representative of three independent experiments.

3.2. Identification of the caspase-3 cleavage sites in PKD1 by microsequencing

Equal amounts of two cleaved fragments (Mr 62,000 and Mr 59,000) were detected by immunoblotting. The caspase-3 recognition site is DXXD with Asp residues at both the P1 and P4 positions [25]. PKD1 has three such sites (DDND^{355}S, DDMD^{367}E and DQED^{397}S) between the second cysteine-rich and PH domains (see Fig. 2B), any of which could yield cleaved fragments of approximately the same size as those observed in the immunoblots. To determine the caspase-3 cleavage sites in PKD1, we digested recombinant GST-PKD1 with caspase-3, and the cleavage products were separated by SDS-PAGE and transferred to PVDF membrane. After Amido Black staining, the bands were excised (Fig. 2A) and subjected to N-terminal degradation. Three major bands were observed: F59 and F62, as already detected by immunoblot analysis, plus an additional band with an apparent mass of 100 kDa (F100).

N-terminal sequencing of these three bands yielded three polypeptide sequences unambiguously identifying D^{355} and D^{397} as the two caspase-3 cleavage sites which generate the F62 and F59 PKD1 fragments, respectively. The third site identified, A^{16}, is not in a classical caspase-3 consensus sequence and may reflect unspecific cleavage.

3.3. Processing of PKD1 by different caspases

Purified recombinant GST-PKD1 was incubated for 2 h at 37 °C with recombinant active caspases-1, -2, -3, -6, -7, -8 and -11 (Fig. 3). Under the conditions used, caspases-3, -6 and -11 produced F100, F62 and F59 fragments; caspase-7 produced F62 and F59 only, and caspase-1 produced F59, whereas caspase-8 generated F100 and F59. Two alternative fragments with relative molecular masses of 60,000 and 63,000 were observed with caspases-1 and -8, respectively. Caspase-2 processed GST-PKD1 rather poorly. These results suggest that GST-PKD1 is an in vitro substrate for both initiator and effector caspases, and that the processing sites may differ between caspases.

3.4. Doxorubicin induces apoptotic cell death

It was recently reported that ara-C induces apoptosis and cleavage of PKCμ through the activation of caspase-3 in U937 myeloid leukemia cells [11]. In our experimental system using PKD1-overexpressing A431 cells, we could not induce cell death using ara-C treatment. By contrast, doxorubicin, an anthracycline antibiotic, caused extensive apoptotic cell death as visualized by Hoechst staining (Fig. 4A) and quantified by MTT test (Fig. 4B). After treatment with 40 μM doxorubicin, we observed cytochrome *c* release into the cytosol (Fig. 5C) and PARP fragmentation which increased significantly by 4 h and peaked at 6 h (Fig. 5B). This was followed by the appearance of dying cells showing typical apoptotic morphology (e.g., membrane blebbing and cell shrinkage). The cellular level of tubulin remained constant during the entire course of the doxorubicin treatment excluding a necrotic type of cell death (Fig. 5D). All the morphological and biochemical characteristics of apoptosis induced by doxorubicin were prevented by the broad specificity caspase-inhibitor zVAD-fmk.

3.5. PKD1 is cleaved in vivo upon treatment with doxorubicin

To determine whether PKD1 is cleaved during doxorubicin-induced caspase activation and apoptosis, we treated PKD1-overexpressing A431 cells with doxorubicin for various times and performed Western blot analysis on cell lysates using the anti-C terminal PKD1 antibody (Fig. 5A). The resulting PKD1 fragments were of the same size as those found in our in vitro experiments (F59 and F62). Using the optimal 40 μM doxorubicin dose, we detected PKD1 cleavage as early as 4 h after initiating cell treatment. The kinetics

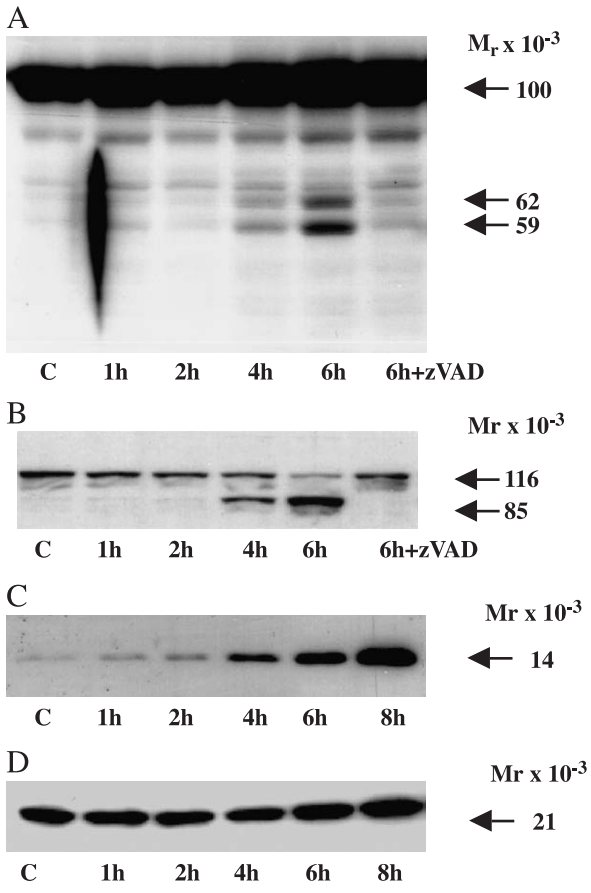

Fig. 5. Doxorubicin-induced PKD1 cleavage is preceded by cytochrome *c* release and coincided with PARP fragmentation. PKD1-overexpressing A431 cells were treated with or without 40 μM doxorubicin for the indicated times, then lysed for Western blotting using PKD1 antibody (A), PARP antibody (B), cytochrome *c* antibody (C) or tubulin antibody (D). In experiments (A) and (B), a 1-h preincubation with 100 μM zVAD was performed where indicated. The results are representative of three independent experiments.

Table 1
Time-dependent activation of PKD1 by doxorubicin

Time (h)	0	1	2	4	6	6 + zVAD
% Activity	100	132	141	129	297	145

A431-PKD1 cells were incubated with 40 μM doxorubicin for the indicated times. Where indicated, cells were pretreated cells with 100 μM zVAD for 1 h. Cells were lysed and immunoprecipitated with anti-PKD mix antibody. PKD1 was eluted from the immunoprecipitates with the immunizing peptide, and eluted PKD1 activity was measured in a syntide-2 kinase assay. The results are representative of three independent experiments and are expressed as % activity of control.

of PKD1 cleavage coincided with doxorubicin-induced PARP fragmentation (Fig. 5B) suggesting a dependence on caspases. Indeed, pretreatment of cells with zVAD-fmk, a general caspase inhibitor, blocked both PARP fragmentation (Fig. 5B; 6 h + zVAD) as well as cleavage of PKD (Fig. 5A; 6 h + zVAD).

3.6. Activation of PKD1 upon treatment with doxorubicin

Cells were treated with doxorubicin to examine the effect of this drug on PKD1 activation. The activity of immunoprecipitated PKD1 was measured with syntide-2 as substrate (Table 1). The appearance of PKD1 fragments and the activation of PKD1 followed similar kinetics (compare Fig. 5A and Table 1). Furthermore, pretreating cells with zVAD-fmk prevented the activation of PKD1. These findings support the proposal that doxorubicin-induced caspase activation leads to PKD1 cleavage and activation.

4. Discussion

In this study, we focused on the mechanism by which PKD1 is regulated in apoptotic conditions, and we demonstrated, both in vitro and in vivo, that PKD1 is cleaved by caspases, and that this cleavage is associated with an increase in kinase activity.

Two lines of evidence suggest that caspase-3 or a caspase-3-like protease can mediate PKD1 cleavage: firstly, Edman microsequencing showed that DDND^{355}S and DQED^{397}S are the cleavage sites in PKD1 for caspase-3. These sites represent typical caspase-3 cleavage sites. Secondly, processing of PKD1 was prevented by DEVD-fmk or zVAD-fmk, the former compound being a specific caspase-3 inhibitor. In our in vitro experiments, the specific capase-3 inhibitor, DEVD-fmk, completely abolished PKD1 cleavage. By contrast, in vivo, only the general caspase inhibitor, zVAD-fmk, was able to completely block the PKD1 processing and inhibit apoptotic cell death in vivo. This may suggest that the in vivo processing and activation of PKD1 may implicate more than one caspase. Furthermore, we established that, during apoptosis induced by treatment with doxorubicin, PKD1 is cleaved, generating two catalytic fragments designated as F59 and F62.

Our results differ from the results reported by Endo et al. [11] who treated U-937 myeloid leukemia cells with several genotoxic agents to induce apoptosis and observed PKCμ cleavage. These authors reported that caspase-3-mediated cleavage of PKCμ produced a Mr 60,000 catalytic fragment both in vivo and in vitro, and suggested that the unconventional CQND^{378}S site was the caspase-3 cleavage site in PKCμ. However, the suggested cleavage site of PKCμ (CQND^{378}S, underlined in Fig. 2B) is not a consensus sequence for caspase-3 and is not conserved in any of the aligned PKD1 sequences in Fig. 2B. PKD1, the mouse homolog of PKCμ, contains three potential DXXD caspase-3 cleavage sites, namely, DDND^{355}S, DDMD^{367}E and DQED^{397}S. Two of these, DDND^{355}S and DQED^{397}S, are also present in PKCμ (Fig. 2B). Moreover, the DXXD^{355} site is conserved in PKD1 in a wide range of species (human, mouse, rat, chicken and pufferfish), and the DXXD^{397} site is conserved among all mammalian species observed . Here, we provide in vivo and in vitro experimental evidence, using Western blotting and microsequencing, to show that PKD1 is cleaved by caspases at the sites conserved between PKD and PKCμ [11]. Previous results by Endo et al. suggested the possibility of PKD activation upon cleavage by caspases in vivo. Here, we show that PKD1 is indeed activated in vivo through a caspase-dependent cleavage upon treatment of cells with doxorubicin. The location of the caspase cleavage sites at the region between the regulatory and catalytic domain confirms the idea that removal of the regulatory zinc finger domains alone can induce PKD1 activation without phosphorylation by upstream kinases. This hypothesis is supported by the results of deletion analysis of PKD [26]. There are multiple examples of both up- and down-regulation of kinase activity after cleavage by caspases: Mst1[27], γ-PAK [28]and PKN [29] are cleaved and activated by a caspase-3 like protease. It is known that certain PKC isoforms are also cleaved by caspases and are either activated like PKCθ [30] and PKCδ [31] or first activated and later degraded by the proteasome like PKCξ [32].

In conclusion, proteolytic activation of PKD1 at two alternative cleavage sites represents a novel mechanism of PKD1 regulation. The F59 and F62 PKD1 catalytic fragments still contain the full PH domain which may mediate specific interactions with proteins or small ligands and determine the intracellular relocalization of the kinase domain-containing fragment [33,34].

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