CPP32 activation during dolichyl phosphate-induced apoptosis in U937 leukemia cells

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Received 21 April 1997; revised version received 4 June 1997

Abstract Treatment of U937 cells with dolichyl phosphate led to an increase in the activity of the ICE family protease CPP32, accompanied with cleavage of pre-CPP32 to generate p17. Peptide inhibitors YVAD-cmk and Z-Asp-CH_2-DCB (specific to ICE) and DEVD-CHO (specific to CPP32) blocked the dolichyl phosphate-induced apoptosis. The dolichyl phosphate-induced increase of CPP32 activity was inhibited by adenylate cyclase inhibitors, SQ 22536 and 2′,5′-dideoxyadenosine. Dolichyl phosphate caused a transient increase of intracellular cAMP concentration. The results suggest that modulation of cAMP synthesis due to the stimulation of adenylate cyclase by dolichyl phosphate plays a critical role in CPP32 activation and apoptosis.

Key words: CPP32; Protease activity; Apoptosis; Inhibitor; Adenylate cyclase; Western blotting

1. Introduction

Recent studies have strongly implicated the interleukin-1β-converting enzyme (ICE)/CED-3 family of cysteine proteases as key participants in the apoptotic cell death that can occur during development [1,2], due to ligation of the FAS (CD95) or tumor necrosis factor (TNF) receptors [3–7], trophic factor deprivation [8] and a number of other apoptosis-inducing stimuli [9–11]. In mammals, the ICE/CED-3 family includes several members, which have been divided into at least three subfamilies with homology to the prototype proteases ICE, CPP32, and Ich-1 [12]. The protease subfamilies have distinct substrate specificities [13], and different kinetics of activation after delivery of an apoptotic signal [14]. The effects of these proteases in apoptosis appear to be manifested via the cleavage of key homeostatic cellular substrates, including poly (ADP-ribose) polymerase [9] and RB protein [15].

Previously we found that dolichyl phosphate induces apoptotic changes, including nuclear fragmentation and internucleosomal cleavage of genomic DNA [16]. However, the mechanism of intracellular delivery of the dolichyl phosphate-induced apoptotic signal is unknown. In the present study, we have examined the effect of dolichyl phosphate on an ICE/CED-3 family cysteine protease, CPP32. Since dolichyl phosphate activated CPP32, we then examined the effects of adenylate cyclase inhibitors, such as SQ 22536 and 2′,5′-dideoxyadenosine (DDA). The results together with the transient increase of adenosine 3′,5′-cyclic monophosphate (cAMP) suggest that stimulation of cAMP synthesis plays an important role in CPP32 activation and in the dolichyl phosphate-induced apoptotic pathway.

2. Materials and methods

2.1. Materials

Dolichyl phosphate was kindly provided by the Tsukuba Research Laboratories of Eisai Company. Acetyl-Asp-Glu-Val-Asp-α-(4-methylcoumaryl-7-amide) (DEVD-MCA), acetyl-Tyr-Val-Ala-Asp-α-(4-methylcoumaryl-7-amide) (YVAD-MCA) and 7-amino-4-methylcoumarin (AMC) were purchased from Peptide Institute, Inc. (Osaka, Japan). ICE inhibitor II (Ac-Tyr-Val-Ala-Asp-chloromethylketone; YVAD-cmk), ICE inhibitor III (benzyloxycarbonyl-Asp-CH_2OC(O)-2,6-dichlorobenzene; Z-Asp-CH_2-DCB), apopain (CPP32) inhibitor (Ac-Asp-Glu-Val-aspartic acid aldehyde; DEVD-CHO) and DNA molecular weight standard pHY marker were from Takara (Kyoto, Japan). 9-(Tetrahydro-2-furanyl)-9H-purin-6-amine (SQ 22536) was from Research Biochemicals International (Maine, USA), 2′,5′-dideoxyadenosine (DDA) from Calbiochem Novabiochem (California, USA). Mouse monoclonal anti-CPP32 antibody was from Transduction Laboratories (Kentucky, USA). Cyclic AMP EIA system was from Amersham (Little Chalfont, England).

2.2. Cell culture and induction of apoptosis

Human promonocytic leukemia U937 cells were routinely cultured at 37°C in RPMI 1640 containing 10% fetal calf serum, using standard cell culture procedures. Cells were washed and resuspended at 2×10^6 cells/ml in the same medium, and cultured in the absence or presence of dolichyl phosphate dissolved in ethanol:dodecane (98:2, v/v). The concentration of ethanol:dodecane was 1% in the culture medium and the presence of the solvent caused neither CPP32 activation nor DNA fragmentation. Cells were incubated for the indicated time at 37°C, collected, and washed with ice-cold phosphate-buffered saline.

2.3. Inhibitors

The effect of protease inhibitors (YVAD-cmk, Z-Asp-CH_2-DCB and DEVD-CHO) and adenylate cyclase inhibitors (SQ 22536 and DDA) on dolichyl phosphate-induced apoptosis were evaluated. YVAD-cmk and Z-Asp-CH_2-DCB were dissolved in dimethyl sulfoxide (DMSO). DEVD-CHO, SQ 22536 and DDA were dissolved in distilled water. The final concentration of DMSO was less than 0.2%, a level which was confirmed to have no influence on the experimental outcome. Inhibitors were added to the medium at the indicated time prior to the treatment with dolichyl phosphate.

2.4. Analysis of DNA fragmentation by agarose gel electrophoresis

DNA from whole cell populations, incubated in the presence or absence of dolichyl phosphate and/or protease inhibitor for 8 h, was

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PII S0014-5793(97)00763-1

extracted and electrophoresed on 1. 7% Nusieve:agarose (3:1) gels as described in our previous paper [17]. Each experiment was repeated at least three times.

2.5. CPP32- and ICE-like protease assays

Cysteine protease activity was measured using a modified procedure of Walker et al. [18]. Briefly, cells were lysed in buffer containing Tris-HCl (pH 7.5) and 0.2% Triton X-100 for 30 min on ice, and clarified by centrifugation. Enzyme reaction mixtures contained 50 μM fluorescent substrate, DEVD-MCA or 100 μM YVAD-MCA and the cell lysate (approximately 150 μg protein). The reaction performed in 50 mM Tris-HCl (pH 7.0), 0.5 mM EDTA, 1 mM dithiothreitol, 20% glycerin and 0.1% Triton X-100. Fluorescent AMC product formation was measured with excitation at 380 nm and emission at 460 nm using a Cytofluor II plate reader (Perseptive Biosystems). Protease activity was expressed as AMC cleaved per mg protein per minute.

2.6. Western blotting

For anti-CPP32 immunoblots, cell lysates (50 μg protein/lane) were separated on 14% SDS-polyacrylamide gels under reducing conditions, transferred to nitrocellulose membrane, and probed with mouse monoclonal anti-CPP32 followed by goat anti-mouse antibody coupled to horseradish peroxidase. Products were visualized using the ECL system (Amersham).

2.7. cAMP assay

Cyclic AMP was extracted and quantitated by enzyme immunoassay system with anti-cAMP antibody, according to the manufacturer's instructions. Cyclic AMP was extracted from cells by ice-cold 65% ethanol. The extracted material was dried under the stream of argon gas. Cyclic AMP content was expressed as fmol of cAMP per 10^6 cells.

3. Results and discussion

3.1. Effect of inhibitors of ICE/CED-3 family proteases on dolichyl phosphate-induced DNA fragmentation

When U937 cells were continuously treated with 20 μg/ml of dolichyl phosphate, more than 90% of the cells died within 10 h, as determined by using the WST (water soluble-tetrazolium salt) assay method, an improved MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) cell proliferation assay (data not shown). DNA fragmentation characteristic of apoptosis occurred within 8 h after the treatment with dolichyl phosphate (Fig. 1). To examine the involvement of ICE/CED-3 family proteases in the induction of apoptosis by dolichyl phosphate, we studied the effect of inhibitors of ICE/CED-3 family proteases on the DNA fragmentation induced by dolichyl phosphate. The inhibitors used were a peptide chloromethylketone YVAD-cmk, an aspartate-based protease inhibitor Z-Asp-CH_2-DCB and an aldehyde DEVD-CHO. YVAD-cmk [19], which was developed initially as an irreversible ICE inhibitor, can also inhibit CPP32 and other ICE-like proteases [9]. Z-Asp-CH_2-DCB can inhibit ICE and other proteases which cleave the residues next to Asp, including Ich-1 and granzyme B [20]. DEVD-CHO inhibits CPP32 as well as several ICE/CED-3 family members, including MCH3, which is closely related to CPP32 [21]. All of these inhibitors showed a very efficient protection of the cells from dolichyl phosphate-induced DNA fragmentation (Fig. 1) and morphological changes typical of apoptosis (data not shown). Treatment of U937 cells with DEVD-CHO, YVAD-cmk or Z-Asp-CH_2-DCB at the same concentration did not cause DNA fragmentation. The inhibitory effect of YVAD-cmk in this paper is in contrast to the report showing that apoptosis of U937 cells caused by anti-FAS and TNF-α cytotoxicity is not prevented by YVAD-cmk [6]. These data suggest that CPP32 and/or other ICE-like proteases are involved in apoptotic cell death induced by dolichyl phosphate, and that the signal transduction cascade of dolichyl phosphate is, at least in part, different from those associated with apoptosis induced by anti-FAS or TNF.

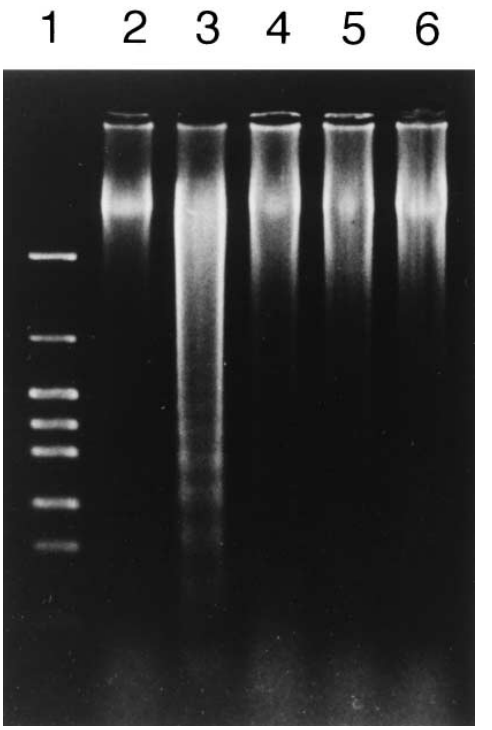

Fig. 1. Effect of inhibitors of ICE-like proteases on dolichyl phosphate-induced DNA cleavage. Cells were incubated at 37 for 2 h in either control medium (lanes 2 and 3), or in medium supplemented with either 600 μM YVAD-cmk (lane 4), 200 μM Z-Asp-CH_2-DCB (lane 5) or 600 μM DEVD-CHO (lane 6). Then, 15 μg/ml dolichyl phosphate (lanes 3, 4, 5 and 6) or ethanol:dodecane (98:2, v/v) (lane 2) was added and incubation was continued for 8 h. DNA samples were prepared and electrophoresed as described in the text. Lane 1, pHY markers (4870, 2016, 1360, 1107, 926, 658, 489 and 267 bp).

3.2. Dolichyl phosphate-induced CPP32-like activity

Fig. 2 shows that dolichyl phosphate activates CPP32-like activity in a dose- and time-dependent manner. In a time course experiment with dolichyl phosphate at the concentration of 20 μg/ml for up to 5 h, CPP32-like activity was not detected for 1 h, then increased up to 3 h, and thereafter decreased gradually (Fig. 2A). The CPP32-like activity increased with increase of dolichyl phosphate concentration from 2.5 to 7.5 μg/ml. Induction of activity was maximum at the concentration of 10 μg/ml, when the cells were incubated for 3 h (Fig. 2B). Half-maximal induction of CPP32-like activity occurred with about 4 μg/ml dolichyl phosphate. The maximal level of the induction of CPP32-like activity was about 7–8 times that of the control experiment with an equivalent amount of ethanol:dodecane (98:2, v/v).

In contrast to CPP32, ICE-like activity was not detectable in the experiment using 50 or 100 μM YVAD-MCA as a substrate following dolichyl phosphate stimulation of U937 cells. Similar results have been obtained for ICE activity stimulated by FAS in Jurkat cells [22].

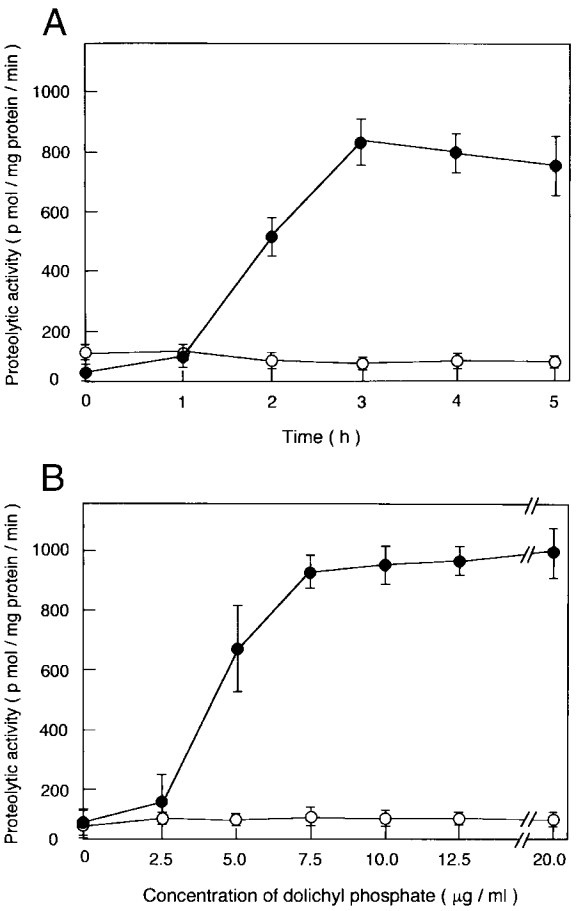

Fig. 2. Time course and dose-response relation of dolichyl phosphate-induced CPP32-like protease activity. (A) Cells were incubated for the indicated times in the presence of dolichyl phosphate (20 μg/ml). (B) Cells were incubated for 3 h in the presence of dolichyl phosphate at the indicated concentration. Protease activity was assayed using 50 μM DEVD-MCA (●) or 100 μM YVAD-MCA (○) as the substrate and expressed as pmol AMC cleaved per mg protein per min. All values are the mean ± S.D. of triplicate determinations made in parallel.

3.3. Dolichyl phosphate-induced CPP32 activation

CPP32 normally exists in cytosol as an inactive precursor and only appears as the active form when cells are undergoing apoptosis. Erhardt and Cooper demonstrated that PI-3 kinase inhibitors (wortmannin and LY294002) and etoposide convert CPP32 into the active p17 subunit, while treatment with TNF-α resulted in the accumulation of p20 subunit in U937 cells [23]. As shown in Fig. 3, 17-kDa peptide was scarcely detectable at 0 h, but detectable after 2 h and was clearly evident after 3 h treatment. Thus, the 32-kDa pro-CPP32 was converted to the 17-kDa peptide characteristic of active CPP32 by dolichyl phosphate treatment.

Table 1
The effects of adenylate cyclase inhibitors on dolichyl phosphate-induced CPP32 activity

	Inhibition of CPP32 activity (%)
SQ 22536	73.0 ± 15.4
DDA	74.5 ± 25.3

Cells were incubated at 37°C in either control medium, or in medium supplemented with 5 mM SQ 22536 or 5 mM DDA for 15 min. Then, dolichyl phosphate (10 μg/ml) was added. CPP32 activity was determined 2 h after dolichyl phosphate treatment and expressed as a percentage of the appropriate control under each experimental condition. Values are the mean ± S.D. of 3–4 different experiments.

3.4. Involvement of adenylate cyclase in dolichyl phosphate-induced CPP32 activation

Inhibitors of adenylate cyclase (SQ 22536 and DDA) were used to examine the participation of the cAMP pathway in dolichyl phosphate-induced apoptosis (Table 1). CPP32 activation induced by dolichyl phosphate was decreased to 25–27% of the original level when cells were preincubated with 5 mM SQ 22536 and 5 mM DDA. Treatment of U937 cells with SQ 22536 or DDA alone had no effect on CPP32 activity. In addition, SQ 22536 or DDA itself (up to 50 μM) did not show any change in CPP32 activity in vitro. We further examined the effect of dolichyl phosphate on cellular cAMP concentrations in U937 cells. As shown in Fig. 4, dolichyl phosphate (15 μg/ml) caused a transient increase in the intracellular levels of cAMP, from a basal level of approximately 500 fmol to greater than 750 fmol/10^6 cells within 60 min. These results suggest that the elevation of intracellular cAMP concentration by dolichyl phosphate is a key event in the induction of apoptosis.

The intracellular level of cAMP can be modulated in several

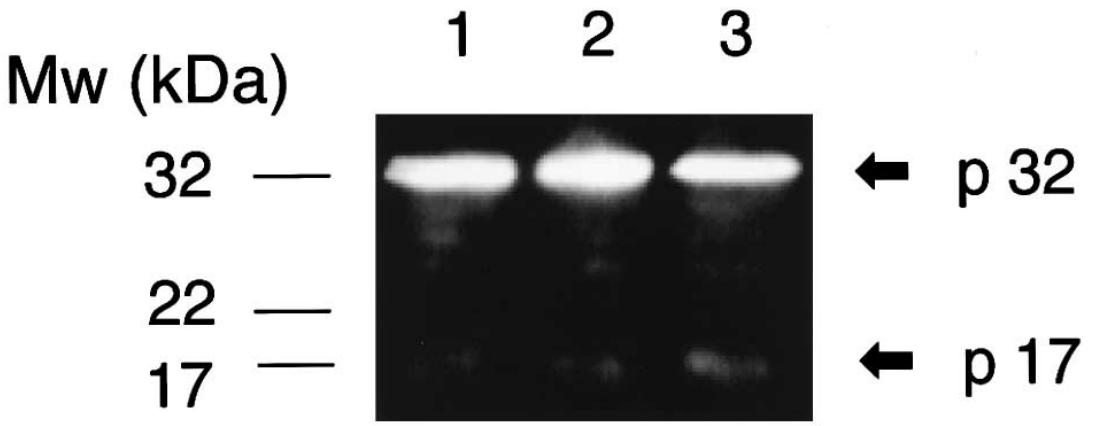

Fig. 3. Time course of dolichyl phosphate-induced CPP32 activation. Cells were incubated for 2 h (lane 2) or 3 h (lane 3) in the presence of dolichyl phosphate (15 μg/ml). Proteins were then extracted and separated by SDS-PAGE, followed by western blotting and probing for anti-CPP 32 antibody. Lane 1, 0 time.

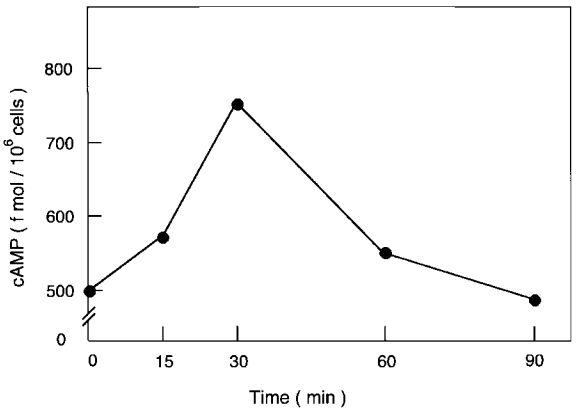

Fig. 4. Time course of dolichyl phosphate-induced modulation of intracellular cAMP levels. Cells were incubated for the indicated times in the presence of dolichyl phosphate (15 μg/ml). Intracellular cAMP levels were measured by enzyme immunoassay and expressed as fmol cAMP per 10^6 cells as described in the text. Results shown are representative of three independent experiments.

ways, i.e. by (i) stimulation of adenylate cyclase with a drug such as forskolin, (ii) increase of cAMP mediated by steroid hormones and PGE1 [24], (iii) accumulation of cAMP through a receptor-coupled mechanism, and (iv) addition of membrane-permeable cAMP analogues. Elevation of cAMP by drugs, such as forskolin [25], gliotoxin [26], erythromycin and dibutyryl cAMP (Bt_2cAMP) [27] leads to apoptotic cell death. In contrast, inhibition of cAMP synthesis by ceramide [28], radiation [29] and serum deprivation [30] also induces apoptotic cell death in a variety of cells. In these cases, the cells can be rescued by the addition of cell-permeable Bt_2cAMP.

It has been shown that the cellular level of dolichyl phosphate and its derivatives may limit cell division by controlling the numbers of functional receptors for growth factors via glycosylation of the receptors [31]. However, we showed previously that inhibitors of the N-linked glycosylation mediated by dolichyl phosphate did not affect the apoptotic cell death [16]. The molecular mechanism by which dolichyl phosphate induces cAMP synthesis is not yet known. It is conceivable that dolichyl phosphate increases the level of intracellular cAMP either by direct interaction with adenylate cyclase or through receptor-mediated mechanisms. Overall, the results imply that cAMP accumulation induced by dolichyl phosphate is an important step in the induction of apoptotic cell death. The fine turning of apoptosis might be regulated by a concerted action of cAMP mediated signals induced by several drugs and antibodies described above and some other mechanisms such as growth factors and hormones which exert their effects in a cAMP independent manner. The precise understanding of the role of the factors and the cross talk systems between the different signals that induce apoptosis remains for further investigation.

Acknowledgements: This work was supported in part by a Sasakawa Scientific Research Grant from The Japan Science Society to Y.Y.

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