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D4-GDI is cleaved by caspase-3 during daunorubicin-induced apoptosis in HL-60 cells

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Abbreviations: Ac-DEVD-CHO, N-acetyl-Asp-Glu-Val-Asp-CHO (aldehyde); Ac-DEVD-AFC, N-acetyl-Asp-Glu-Val-Asp-AFC (7-amino-4-trifluoromethyl-coumarine); Ac-YVAD-CHO, N-acetyl-Tyr-Val-Ala-Asp-CHO (aldehyde); GDI, GDP-dissociation inhibitor; ICAD, an inhibitory protein for the caspase-activated deoxyribonuclease (CAD); ICE, IL-1β-converting enzyme; LPS, lipopolysaccharide; MAPK, mitogen activated protein kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PARP, poly(ADP-ribose) polymerase.

Abstract

Daunorubicin, an anti-cancer drug, is known to induce apoptosis in HL-60 cells in a dose-dependent manner through the activation of caspase-3 (CPP32). Caspase-3 selective inhibitor, Ac-DEVD-CHO, prevented both the activation of caspase-3 and cleavage of poly(ADP-ribose) polymerase (PARP). D4-GDI is a GDP dissociation inhibitor for the Ras-related Rho family GTPase in hematopoietic cells. Here we report that D4-GDI is a substrate for the caspase-3. D4-GDI was cleaved to a 23 kDa fragment by daunorubicin treatment in HL-60 cells with kinetics that parallel the onset of apoptosis. D4-GDI cleavage as well as DNA fragmentation was inhibited by treatment with Ac-DEVD-CHO but not with Ac-YVAD-CHO, a caspase-1 inhibitor. These data suggest that D4-GDI of Rho family GTPase may be regulated during apoptosis through the caspase-3 mediated cleavage of the GDI protein.

Keywords: daunorubicin, caspase-3, D4-GDI, poly(ADP-ribose) polymerase, apoptosis

Introduction

Daunorubicin is widely used in the treatment of acute myeloid leukemia (AML). Despite the extensive and generalized use for more than 30 years, its cytotoxic mechanism remains still obscure. Several hypotheses have been suggested including DNA intercalation, inhibition of topoisomerase II, free radical generation with consequent DNA damage or lipid peroxidation, DNA alkylation and cross-linking, and direct membrane effects (Gewirtz, 1999). Recently, it has been shown that stimulation of ceramide synthesis (Bose *et al.*, 1995; Jaffrezou *et al.*, 1996) or caspase activation (Gamen *et al.*, 1997; Turnbull *et al.*, 1999) mediate daunorubicin-induced apoptosis. Activation of caspase, a family of cysteine protease, has been demonstrated in different pathways of apoptosis (Polverino and Patterson, 1997). Caspase is synthesized as catalytically inactive proenzymes comprising a large and a small subunit with a variable length amino-terminal prodomain. On activation, the prodomain is lost by catalytic cleavage of carboxy-terminal at an aspartate residue, with heterodimerization of the large and small subunits to form the active enzyme (Nicholson and Thornberry, 1997). Numerous substrates for caspase were identified. Those include poly(ADP-ribose) polymerase (PARP) (Lazebnik *et al.*, 1994), lamin (Oberhammer *et al.*, 1994), DNA fragmentation factor (DFF/ICAD) (Liu *et al.*, 1997), and gelsolin (Kothakota *et al.*, 1997). Although many proteins are cleaved during apoptosis, a role of this cleavage has been identified only in a few of them. ICAD is cleaved by caspase-3, which leads to the release of DNase and causes subsequent DNA digestion during apoptotic process (Enari *et al.*, 1998). Cleavage of gelsolin and lamin cause the characteristic morphological changes for apoptosis (Rao *et al.*, 1996; Kothakota *et al.*, 1997).

In resting cells, members of the Rho family exist as an inactive cytosolic complex with a class of regulatory protein known as a GDP dissociation inhibitor (GDI) (Bokoch and Der, 1993; Geyer and Wittinghofer, 1997) and three forms of GDI were identified: RhoGDI-1, RhoGDI-2 (also called D4/Ly-GDI), and RhoGDI-3. D4-GDI is specifically expressed in human and murine hematopoietic tissues, predominately in B- and T-lymphocyte cell lines (Lelias *et al.*, 1993; Scherle *et al.*, 1993). This protein binds to a majority of the Rho family GTPase and keeps the Rho protein in its GDP-bound inactive state incapable of interacting with effector targets or other regulatory

proteins (Bokoch and Der, 1993; Olofsson, 1999). The Rho family of small GTPase regulates many cellular processes including actin polymerization that leads to the formation of stress fibers and focal adhesions (Ridley and Hall, 1992; Sasaki and Takai, 1998), generation of superoxide in activated neutrophils and macrophages (Abo *et al.*, 1991; Guillemot *et al.*, 1996), and the stress response through activation of c-Jun N-terminal kinase and p38 MAPK2 (Coso *et al.*, 1995; Minden *et al.*, 1995; Zhang *et al.*, 1995; Narumiya, 1996).

It has been reported that D4-GDI is cleaved by caspase-1 in LPS- or nigercin-treated inflammatory cells (Danley *et al.*, 1996). D4-GDI is also reported to be cleaved by caspase-3 during apoptosis in Jurkat T-cells treated with anti-Fas antibody, staurosporine (Na *et al.*, 1996), and overexpressed Bax (Xiang *et al.*, 1996), in BL60 Burkitt lymphoma cells treated with anti-IgM (Rickers *et al.*, 1998) and taxol (Essmann *et al.*, 2000), and in polymorphonuclear neutrophils treated with TNF-α (Kettritz *et al.*, 2000). In this report, we add the observations that D4-GDI is a substrate of caspase-3 in HL-60 cells after induction of apoptosis by daunorubicin. By using cell permeable peptide inhibitors, we were able to demonstrate that caspase-3 is involved in D4-GDI cleavage and apoptosis after treatment with daunorubicin.

Materials and Methods

Culture conditions

HL-60 cells, human leukemia cell line was purchased from American Type Culture Collection. Cells were placed into 75 cm^2 tissue culture flasks and grown at 37°C under a humidified, 5% CO_2 atmosphere in RPMI 1640 medium (Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum and 2 mM glutamine, 10,000 units/ml penicillin, 10 mg/ml streptomycin, and 2.5 μg/ml amphotericin B.

Assay for cell viability

General viability of cultured cells was determined by reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan (Oez *et al.*, 1990). After 48 h incubation with daunorubicin, cells (10^4 cells/well) in 96 well plates were washed twice with phosphate-buffered saline (PBS). MTT (100 μg/0.1 ml PBS) was added to each well. Cells were incubated at 37°C for 1 h, and dimethyl sulfoxide (DMSO) was added to dissolve the formazan crystals. The absorbance was measured at 570 nm with a model Spectra MAX PLUS (Molecular Devices, Sunnyvale, CA, USA).

Caspase activity assay

After treatment with daunorubicin (Sigma, St. Louis, MO, USA), cells were washed with ice-cold PBS and lysed in Triton X-100 buffer (0.5% Triton X-100, 10 mM EDTA, and 10 mM Tris-HCl, pH 7.5) for 30 min on ice. Cell lysates were mixed with caspase assay buffer (10% glycerol, 2 mM DTT, and 20 mM HEPES, pH 7.5) containing caspase substrate, 20 mM Ac-DEVD-AFC (Pharmingen, San Diego, CA, USA), and incubated for 1 h at 37°C. AFC released by caspase was monitored using a spectrofluorometer with an excitation wavelength of 400 nm and an emission wavelength of 505 nm.

Western blot analysis of PARP and D4-GDI cleavage

Cell extracts were separated by gel electrophoresis on a reducing SDS-polyacrylamide gel. Subsequently, the proteins were transferred onto a nitrocellulose membrane (Millipore, Bedford, MA, USA) using a semi-dry blotting apparatus (Bio-Rad, Munich, Germany). Prior to incubation with the PARP (Transduction Lab, Lexington, KY, USA) and D4-GDI antibodies (Pharmingen), the membrane was blocked with 2% BSA for 30 min. After washing, the proteins were detected with an alkaline-phosphatase coupled secondary antibody.

Detection of DNA fragmentation by gel electrophoresis

Cell pellets (3×10^6 cells) were resuspended in 500 μl of lysis buffer (0.5% Triton X-100, 10 mM EDTA, and 10 mM Tris-HCl, pH 8.0) at room temperature for 15 min and centrifuged at 16,000 *g* for 10 min. DNA was then extracted twice with phenol/chloroform (1 : 1), precipitated with ethanol, and resuspended in Tris/EDTA buffer (10 mM Tris-HCl, pH 8.0, and 1 mM EDTA). DNA was analyzed after separation by gel electrophoresis (2% agarose).

Protein determination

Protein concentrations were determined by the method of Bradford (Bradford, 1976) with bovine serum albumin as the standard. All of the samples were assayed in triplicate.

Statistical analysis

Statistical analysis of the data was performed with an unpaired Student's *t*-test. Differences with $P < 0.05$ were considered statistically significant.

Results

Dose-dependent reduction of cell survival

MTT conversion assay was used to determine the viability of HL-60 cells exposed to daunorubicin. As shown in Figure 1, about 40% of cells were viable after exposure to 250 nM daunorubicin for 48 h. The IC_{50} of daunorubicin for cell viability was 200 nM.

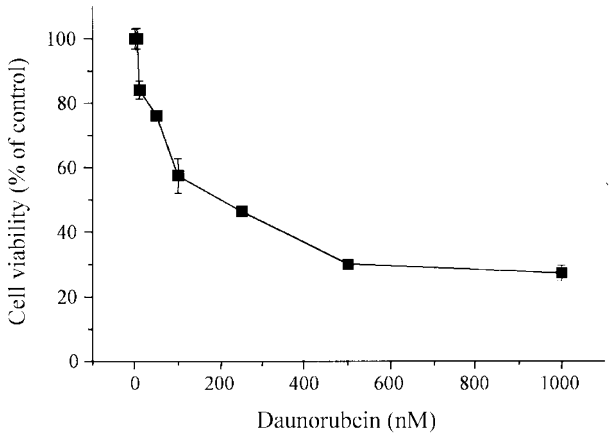

Figure 1. Effect of daunorubicin on cell viability. HL-60 cells were treated with daunorubicin for 48 h and its viability was determined by MTT assay. The percentage of cell viability was calculated as a ratio of A_{570} of control cells (treated with 0.05% PBS vehicle). Each value is the mean ± S.E. of four independent experiments.

Activation of caspase-3 during daunorubicin-induced apoptosis

DNA fragmentation and membrane changes were generally used as markers of apoptotic cell death. However, many researchers consider caspase activation as an even more reliable hallmark of apoptosis than DNA alterations (Nagata, 1997). Until now, several families of caspase have been reported and the caspase-3 is well known in association with apoptosis (Nicholson and Thornberry, 1997). To address the involvement of caspase-3 in daunorubicin-induced apoptosis, Ac-DEVD-AFC, fluorogenic caspase-3 substrate was used for caspase activity assay. As shown in Figure 2, daunorubicin caused dose- dependent activation of caspase-3. However, daunorubicin did not induce the cleavage of Ac-YVAD-AFC, the caspase-1 substrate (data now shown). This protease activity was increased from 12 h and persisted until 24 h (Figure 2B), and reached its submaximal levels at 250 nM after 24 h treatment (Figure 2A). This result was confirmed by Western blotting of PARP, a well- known caspase-3 substrate. Treatment with 250 nM daunorubicin induced proteolytic cleavage of PARP, with the accumulation of the 85 kDa cleaved products in a time dependent manner (Figure 2C).

Western blot analysis of D4-GDI cleavage

To determine the participation of D4-GDI cleavage in daunorubicin-induced apoptosis, D4-GDI cleavage was investigated by Western blot analysis using a rabbit polyclonal antibody. Figure 4 showed that D4-GDI cleavage was induced after 12 h of incubation with daunorubicin (Figure 4A). In our model system, the time course of D4-GDI cleavage was similar to those of caspase activation and PARP cleavage. The 28 kDa

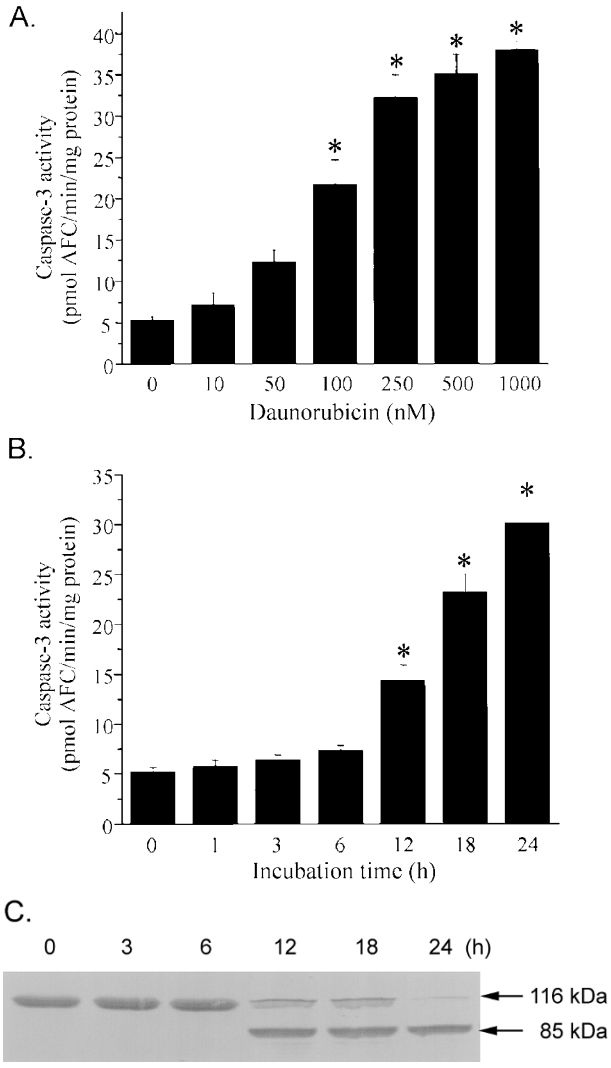

Figure 2. Dose- and time-dependent activations of caspase-3 after daunorubicin treatment. HL-60 cells were treated with either a range of concentrations (0-1000 nM) of daunorubicin for 24 h (**A**), or 250 nM daunorubicin for the indicated time periods (**B**). Cytosolic extracts were prepared and assayed for caspase-3 activity as described in Materials and Methods. Values represent mean ± S.E. of six separate experiments. (Significant difference; $^{*}P<0.05$, $^{**}P<0.01$). (**C**) HL-60 cells were treated with 250 nM daunorubicin for indicated periods and the cleavage of PARP was analyzed by Western blotting as described in Materials and Methods.

D4-GDI band was specifically cleaved to a 23 kDa fragment in a dose-dependent manner (Figure 4B).

Inhibition of apoptogenic signaling events by caspase-3 inhibitor

DNA extract from HL-60 cells incubated with 250 nM daunorubicin for 48 h generated a characteristic ladder pattern of discontinuous DNA fragments on agarose gel electrophoresis (Figure 5, lane 2). When HL-60 cells were pretreated with 3 μM Ac-DEVD-CHO, a selective caspase-3 inhibitor, both caspase-3 activation and PARP

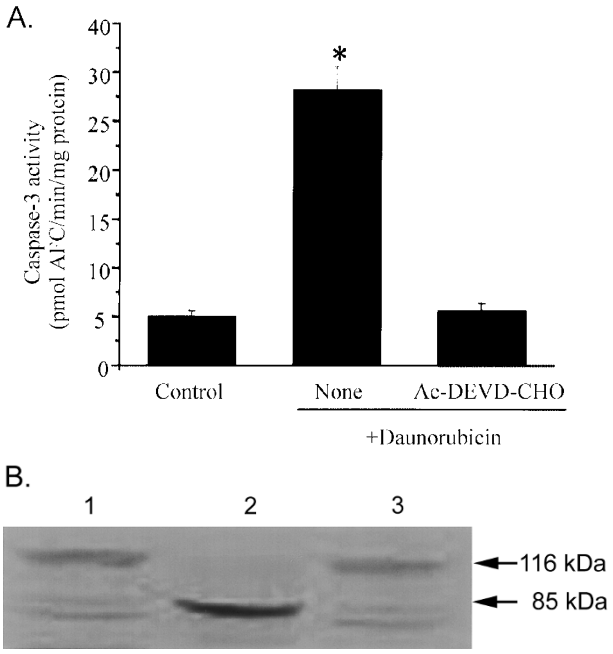

Figure 3. Inhibition of caspase activity by caspase-3 inhibitor. HL-60 cells were preincubated with 3 μM Ac-DEVD-CHO for 3 h followed by treatment with 250 nM daunorubicin for further 24 h. Cytosolic extracts were prepared, and assayed for caspase-3 activity (**A**) or PARP cleavage was analyzed by Western blotting (**B**). Lane 1, control; Lane 2, 250 nM daunorubicin; and Lane 3, 250 nM daunorubicin with 3 μM Ac-DEVD-CHO

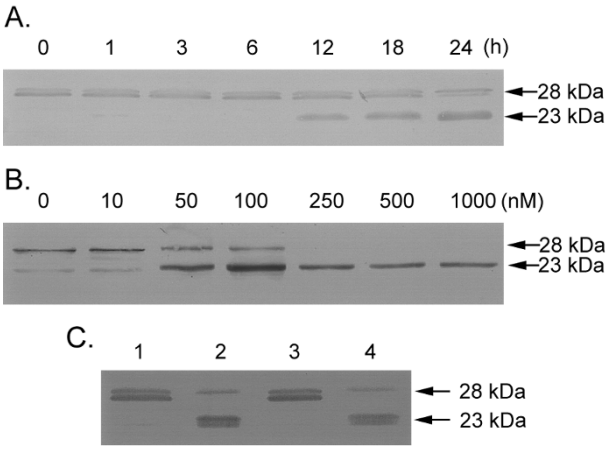

Figure 4. Identification of D4-GDI as a substrate of caspase-3. HL-60 cells were treated with 250 nM daunorubicin for various times (**A**), or a range of concentrations (0-1000 nM) of daunorubicin for 24 h (**B**), and the cleavage of D4-GDI was analyzed by Western blotting. To determine the responsible caspase for D4-GDI, HL-60 cells were pretreated with Ac-DEVD-CHO (3 μM) or Ac-YVAD-CHO (25 μM) for 3 h and followed by treatment with 250 nM daunorubicin for further 24 h (**C**). Lane 1, control; Lane 2, 250 nM daunorubicin; Lane 3, 250 nM daunorubicin with 3 μM Ac-DEVD-CHO, and Lane 4, 250 nM daunorubicin with 25 μM Ac-YVAD-CHO. This experiment was repeated three times with similar results.

cleavage were completely abolished (Figure 3). Furthermore, the caspase-3 inhibitor showed substantial inhibition of both D4-GDI cleavage (Figure 4, lane 3) and apoptosis at concentrations as low as 3 μM (Figure 5, lane 3). The same treatment of HL-60 cells with caspase-1 inhibitor up to 25 μM did not inhibit daunorubicin-induced D4-GDI cleavage (Figure 4C, lane 4) and apoptosis (Figure 5, lane 4). These results provide the evidence that caspase-3 is required for both D4-GDI cleavage and apoptosis, but caspase-1 is not involved.

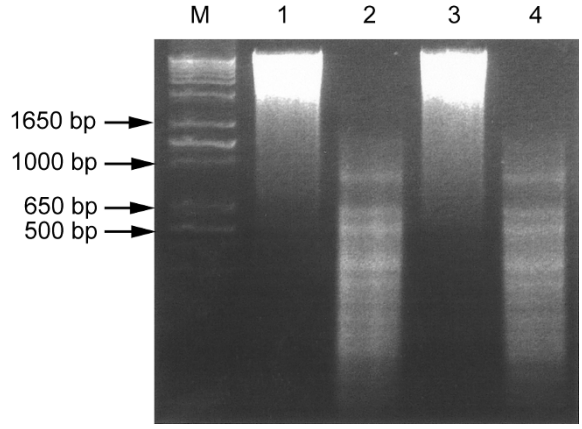

Figure 5. Inhibition of daunorubicin-induced DNA fragmentation by caspase-3 inhibitor. HL-60 cells were treated with daunorubicin for 48 h in the presence of Ac-DEVD-CHO or Ac-YVAD-CHO. DNA was extracted and analyzed by 2% agarose gel electrophoresis as described in Materials and Methods. Lane 1, control; Lane 2, 250 nM daunorubicin; Lane 3, 250 nM daunorubicin with 3 μM Ac-DEVD-CHO, and Lane 4, 250 nM daunorubicin with 25 μM Ac-YVAD-CHO.

Discussion

D4-GDI, initially identified by its homology to Rho-GDI, functions as a negative regulator of Rho family GTPase. Its expression is mainly restricted to the cells of the hematopoietic system including bone marrow, thymus, spleen and lymph nodes (Lelias *et al*., 1993; Scherle *et al*., 1993). Actually, D4-GDI knockout mice were viable with normal thymocyte selection, lymphoid development, and immune responses (Yin *et al*., 1997). The mice did, however, show decreased apoptosis of lymph node cells after IL-2 withdrawal, which implicated the regulation of the Rho GTPase by D4-GDI in lymphocyte survival (or apoptosis) and responsiveness.

We implicated caspase-3 as a possible mediator for D4-GDI cleavage in daunorubicin-treated HL-60 cells undergoing apoptosis. Caspases are cystein-containing, aspartate-specific proteases and key apoptosis mediators in many cells (Kim *et al*., 2000; Kwon *et al*. in press). Caspases can be divided into two major subgroups on the basis of their substrate specificity, sequence homology, and biochemical function: caspase-1 (ICE) like (caspase-1, -4, and 5) and caspase-3 like (caspase-2, -3, and -10) proteases (Nicholson and Thornberry, 1997).

While caspase-1 seems to be mainly involved in inflammation (Cohen, 1997) and partially relating to apoptosis (Suzuki *et al.*, 1998), the caspase-3 plays an important role in apoptosis.

D4-GDI is cleaved from a 28 kDa to a 23 kDa species and it contains two cleavage sites: a caspase-3 cleavage site and caspase-1 cleavage site. Na *et al.* (Na *et al*., 1996) reported that cleavage of D4-GDI and apoptosis could be inhibited by the addition of the caspase-1 inhibitor, Ac-YVAD-CMK in Fas-treated Jurkat T cells. In their *in vitro* experimental system, the authors also showed that incubation of D4-GDI with recombinant caspase-3 resulted in the cleavage of D4-GDI after aspartate and could be blocked by the addition of caspase-3 inhibitor. To confirm the cleavage site in daunorubicin-treated HL-60 cells, cells were pretreated with cell-permeable peptide inhibitors, and then D4-GDI cleavage and apoptosis were studied. We used Ac-DEVD-CHO or Ac-YVAD-CHO, which irreversibly inhibits caspase-3 and caspase-1 activities, respectively. In our experimental system, caspase-3 inhibitor, but not caspase-1 inhibitor, protected daunorubicin-induced caspase activation, PARP cleavage, D4-GDI cleavage, and apoptosis. This is the first report that D4-GDI cleavage is induced by daunorubicin and specifically cleaved by activated caspase-3 in HL-60 cells.

Many proteins are cleaved during apoptosis, but only a few of these substrates have shown to be related with apoptotic process. Activation of caspase-3 cleaves PARP, a well-known substrate. This cleavage leads to its inactivation to prevent futile DNA repair cycles (Lazebnik *et al*., 1994). Although PARP is not essential for cell viability, the cleavage of PARP is another hallmark of apoptosis (Tewari *et al.*, 1995). Treatment of HL-60 cells with 250 nM daunorubicin induced proteolytic cleavage of 116 kDa PARP, with accumulation of the 85 kDa cleaved products. PARP cleavage was apparent at 12 h after daunorubicin treatment with similar time course of caspase activity and preceded DNA fragmentation.

Several proteins have been shown to translocate to the nucleus in different models of apoptosis. In the apoptotic pathway, caspases could degrade ICAD and cause its dissociation from CAD; CAD could then enter the nucleus to degrade the chromosomal DNA (Enari *et al*., 1998). D4-GDI resides in the cytoplasm of resting cells. Following cleavage by caspase, the 23 kDa form of D4-GDI translocated to the nucleus (Krieser and Eastman, 1999). Further evaluation is needed to define the clear relationship between the nuclear translocation of cleaved D4-GDI and the demise of the cells.

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