Overexpression of Bcl-X_L prevents caspase-3-mediated activation of DNA fragmentation factor (DFF) produced by treatment with the photochemotherapeutic agent BPD-MA

David J. Granville[a,b,*], Huijun Jiang[a], Mary T. An[a], Julia G. Levy[a,c], Bruce M. McManus[b], David W.C. Hunt[a,b]

[a] *QLT PhotoTherapeutics Inc., 520 West 6th Avenue, Vancouver, BC V5Z 4H5, Canada*
[b] *Cardiovascular Research Laboratory, Department of Pathology and Laboratory Medicine, St. Paul's Hospital-University of British Columbia, 1081 Burrard Street, Vancouver, BC V6Z 1Y6, Canada*
[c] *Department of Microbiology and Immunology, Faculty of Science, University of British Columbia, 300-6174 University Blvd., Vancouver, BC V6T 1W5, Canada*

Received 18 November 1997

Abstract Photodynamic therapy (PDT) is a clinically effective cancer treatment. For human promyelocytic leukemia HL-60 cells, cleavage of pro-caspase-3 (CPP32/Yama/apopain) into its proteolytically active subunits rapidly follows the photodynamic treatment of these cells with cytotoxic levels of the photosensitizer benzoporphyrin derivative monoacid ring A and visible light. Cleavage of a recently identified cytosolic 45 kDa protein, DNA fragmentation factor (DFF), is required for endonuclease activation leading to DNA fragmentation. In the present study, DFF was rapidly processed following PDT. Overexpression of the anti-apoptotic Bcl-X_L gene in HL-60 cells prevented PDT-induced caspase activation, DFF cleavage and DNA fragmentation. These results demonstrate for the first time an example of chemotherapeutic drug-induced activation of DFF and its regulation by Bcl-X_L.

Key words: Bcl-X_L; DNA fragmentation factor; Apoptosis; Caspase; Photodynamic therapy; Benzoporphyrin derivative monoacid ring A; HL-60 cell

1. Introduction

It is now established that the degradation of key cellular substrates by caspase family proteases is fundamental to the apoptotic process [1]. The most intensively studied of these proteases, caspase-3 (CPP32/Yama/apopain), resides in the cytosolic fraction of cells as a zymogen and is proteolytically activated in cells undergoing apoptosis [2–4]. Caspase-3 substrates include poly(ADP-ribose) polymerase (PARP) [3], sterol regulatory element binding proteins [5], the U1-associated 70 kDa protein [1], and the catalytic subunit of DNA-dependent protein kinase (DNA-PK_{CS}) [1]. Lui et al. [6] recently identified a protein, DNA fragmentation factor (DFF), which is required for the initiation of endonuclease-mediated DNA fragmentation and can be directly activated by caspase-3 in vitro [6]. DFF is a cytosolic factor consisting of 40 kDa and 45 kDa subunits, of which the 45 kDa portion is cleaved into smaller polypeptides by caspase-3 [6]. This observation directly links caspase-3 activation in apoptotic cells with DNA fragmentation through the activation of DFF [6]. Thus, DFF serves as a component in the executioner phase of apoptosis but does not, to date, exhibit any proteolytic characteristics [6].

Bcl-X functions as a Bcl-2-independent regulator of apoptosis [7]. Boise et al. demonstrated that alternative splicing results in two distinct Bcl-X mRNA species [7]. The protein product of the larger mRNA, Bcl-X_L, encodes a protein comparable in function to the anti-apoptotic proto-oncogene Bcl-2 [7]. The second mRNA species, Bcl-X_S, encodes a protein that promotes apoptotic activity [7]. Bcl-X_L can form ion channels upon insertion into synthetic lipid vesicles or planar lipid bilayers suggesting that Bcl-X_L affects cell survival by influencing the permeability of the intracellular membranes to which it is distributed [8]. Bcl-X_L exists in both soluble and membrane-bound forms [9]. In cells undergoing apoptosis, soluble Bcl-X_L shifts to the membrane-bound form, possibly forming ion channels in mitochondria [9]. Bcl-X_L prevents apoptosis by limiting the availability of cytochrome *c* (cyt *c*) in the cytosol [10]. In coimmunoprecipitation studies, Bcl-X_L associated with cyt *c* but not Bcl-X_S [10]. However, Bcl-X_S can interfere with the binding of cyt *c* to Bcl-X_L, thereby promoting apoptosis [10]. In the apoptotic pathway, Bcl-X_L acts upstream of caspase-3 and overexpression of Bcl-X_L corresponds to an absence of caspase-3 activation in cells treated with various chemotherapeutic agents [11–13].

Photodynamic therapy (PDT) is an approved treatment modality for various types of malignancies and involves the topical or systemic application of a photosensitizing agent followed by illumination with a specific wavelength of light [14]. Photoactivation of the chlorin-type photosensitizer, benzoporphyrin derivative monoacid ring A (BPD-MA), eradicates tumor cells through the generation of reactive oxygen intermediates [15]. BPD-MA accumulates at higher levels in leukemic cells as compared to normal blood mononuclear cells [15,16]. In vitro experiments have shown that photoactivation of BPD-MA can selectively deplete leukemic cells while relatively sparing normal hematopoietic progenitor cells [16,17].

Treatment of human promyelocytic leukemia HL-60 cells with BPD-MA and light rapidly produces DNA fragmentation and apoptotic cell death [18]. Caspase-3, but not caspase-1, activation followed by PARP and DNA-PK_{CS} degradation occurred in HL-60 cells treated with BPD-MA and light [18]. The present report demonstrates two novel findings: (1) DFF is rapidly activated following PDT and (2) Bcl-X_L overexpression in HL-60 cells or pretreatment with the caspase-3 inhib-

*Corresponding author (a). Fax: (1) (604) 875-0001.
E-mail: dgranvil@qlt-pdt.com

PII S0014-5793(97)01616-5

itor Z-DEVD-fmk prevents PDT-mediated DFF activation, caspase-3 activity and DNA fragmentation.

2. Materials and methods

2.1. Reagents

Liposomally formulated BPD-MA was provided by QLT PhotoTherapeutics Inc. (Vancouver, BC). All antibodies, with the exception of rabbit anti-DFF, were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). The DFF antibody was kindly provided by Dr. Xiaodong Wang (University of Texas Southwestern Medical School, Dallas, TX). All other drugs and chemicals, unless specified, were from Sigma Chemical Co. (St. Louis, MO).

2.2. Cell culture

The transfected human promyelocytic leukemia HL-60/neo and HL-60/Bcl-X_L cell lines were generously provided by Dr. Kapil Bhalla (Emory University School of Medicine, Atlanta, GA). Detailed methods used to generate these clones have been described [11]. Bcl-2 and Bax levels were comparable in both transfectants [11]. Cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 4 mM L-glutamine, 1 mM sodium pyruvate, 1 mM HEPES, penicillin (100 U/ml) and streptomycin (100 μg/ml) (Gibco BRL, Burlington, Ont.) and G418 (1 mg/ml) (Geneticin; Life Technologies Inc., Grand Island, NY).

2.3. Photoactivation of BPD-MA

For photoinactivation studies, cells were incubated for a total of 60 min at 37°C with or without BPD-MA (0–100 ng/ml) in RPMI medium with 10% FBS. For caspase-3 inhibition studies, Z-Asp-Glu-Val-Asp-fluoromethylketone (Z-DEVD-fmk) (20 μM) (Enzyme Systems Prod., Dublin, CA) was added to cells during the final 30 min of the BPD-MA incubation period. Cells were then exposed to fluorescent red light (620–700 nm) delivered at a rate of 5.6 mW/cm^2 to give a total dose of 2 J/cm^2. Following light treatment, cells were maintained at 37°C until further analysis.

2.4. Preparation of cellular protein extracts

To obtain cytosolic extracts, cells were treated 1 h after PDT in 1 ml of lysis buffer (1% Nonidet P-40 detergent (NP-40), 20 mM Tris, pH 8, 137 mM NaCl, 10% glycerol) supplemented with 1 mM phenylmethylsulfonyl fluoride, aprotinin (0.15 U/ml), and 1 mM sodium orthovanadate for 20 min on ice as previously described [18].

2.5. Protease assay

A cell-free protease assay was performed by incubating 10 μg of cell lysate protein in 150 μl of reaction buffer (1% NP-40, 20 mM Tris, pH 7.5, 137 mM NaCl, 10% glycerol) containing 100 μM of the caspase-3 substrate (acetyl-Asp-Glu-Val-Asp-amino-4-methylcoumarin (Ac-DEVD-AMC)) (Calbiochem, Cambridge, MA) in 96-well microtiter plates [18]. Lysates were incubated at 37°C for 16 h and cleaved substrate fluorescence levels were determined using a CytoFluor 2350 (PerSeptive Biosystems, Ont., Canada) set at excitation and emission wavelengths of 380 nm and 460 nm, respectively.

2.6. Immunoblot analysis

Detergent soluble proteins (30 μg) were separated by SDS-PAGE in 12% acrylamide gels, under reducing conditions followed by Western blotting as described [18]. Membranes were incubated for 45 min using the following polyclonal antibodies at 1 μg/ml: goat anti-PARP, goat anti-CPP32, rabbit anti-DFF or rabbit anti-Bcl-$X_{S/L}$. Membranes were probed with horseradish peroxidase-labelled anti-goat IgG or anti-rabbit IgG antibodies (1:5000) in PBS, 0.05% Tween 20, 5% (w/v) skim milk powder for 30 min at room temperature. Proteins were detected using the enhanced chemiluminescence detection system (Amersham, Arlington Heights, IL) and bands visualized by autoradiography.

2.7. Analysis of DNA fragmentation

Propidium iodide (PI) fluorescence analysis and flow cytometry were utilized to quantitate subdiploid amounts of cellular DNA as described elsewhere [18–20]. A 3 h post-treatment timepoint was utilized since there was little change in the level of DNA fragmentation at later timepoints (not shown).

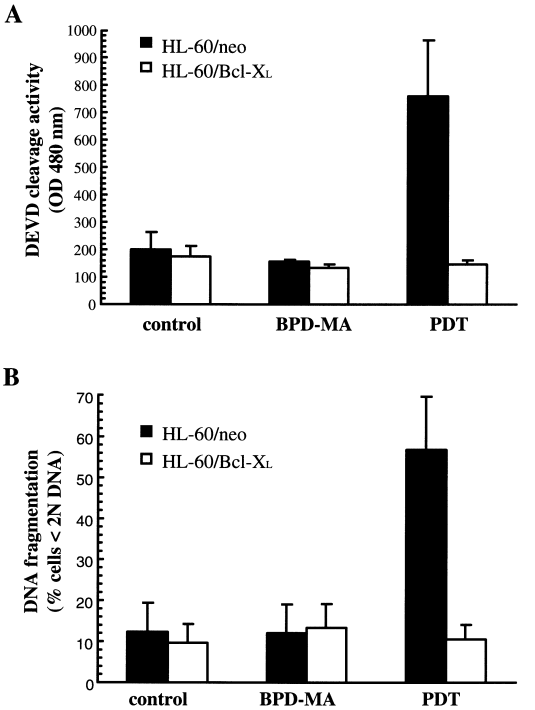

Fig. 1. Overexpression of Bcl-X_L blocks (A) PDT-induced DEVDase activity and (B) DNA fragmentation. A: At 1 h after treatment, lysates for HL-60/neo (■) or HL-60/Bcl-X_L (□) cells were prepared and assayed for their capacity to cleave the fluorescently labelled caspase-3 substrate (Ac-DEVD-AMC). Cytosolic extracts were prepared from cells treated with light (control), BPD-MA (100 ng/ml) or BPD-MA (100 ng/ml) and light (2 J/cm^2) (PDT). B: HL-60/neo (■) and HL-60/Bcl-X_L (□) cells were assessed for DNA fragmentation by PI staining 3 h after the indicated treatments.

3. Results and discussion

3.1. Overexpression of Bcl-X_L blocks caspase-3-like protease activity and DNA fragmentation

We have previously demonstrated that caspase-3 activation, but not caspase-1 activation, occurs in HL-60 cells treated with PDT [18]. Cytosolic extracts prepared from HL-60 cells with overexpression of Bcl-X_L exhibited minimal cleavage of the caspase-3 substrate (Ac-DEVD-AMC) in contrast to the HL-60/neo cells treated with the same level of BPD-MA and light, thereby providing support that Bcl-X_L overexpression blocks caspase-3-like activity (Fig. 1A).

HL-60/neo and HL-60/Bcl-X_L cells were also assessed for the presence of DNA fragmentation 3 h after photoactivation of BPD-MA (Fig. 1B). The vast majority of HL-60/neo cells treated with BPD-MA and light exhibited DNA fragmentation as compared to untreated cells or cells exposed to BPD-MA alone. However, there was no evidence of a change in DNA fragmentation levels in HL-60/Bcl-X_L cells treated with BPD-MA and light at 3 h after photoactivation.

3.2. DFF is rapidly processed following PDT

Since levels of caspase-3-like activity as determined by the protease assay directly corresponded to subsequent levels of DNA fragmentation, our next aim was to examine possible links between these two events. Recent experiments by Liu et al. [6] identified a novel protein (DFF) in staurosporine-treated HeLa cells that is proteolytically cleaved during apoptosis. It was demonstrated that caspase-3 could cleave this

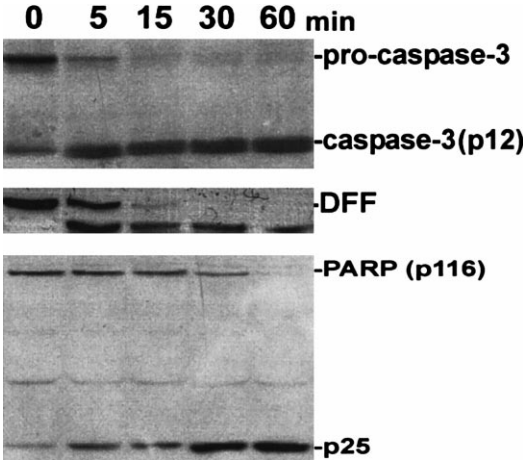

Fig. 2. DFF is rapidly cleaved following PDT. Following PDT, wild type HL-60 cells were lysed at the indicated timepoints. Cell lysates were separated by SDS-PAGE followed by Western immunoblotting using anti-caspase-3 (p12), anti-DFF, or anti-PARP (p25) antibodies.

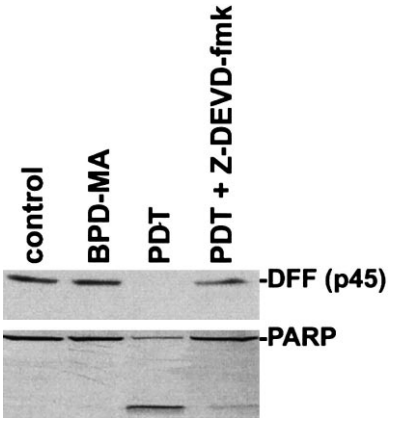

Fig. 3. DFF is cleaved by a caspase-3-like protease. At 1 h after PDT, cell lysates were prepared. Proteins were separated by SDS-PAGE followed by Western immunoblotting using anti-DFF or anti-PARP antibodies. HL-60 cells were treated with light (control), BPD-MA (100 ng/ml), BPD-MA (100 ng/ml) and light (2 J/cm^2) (PDT) or BPD-MA (100 ng/ml) and Z-DEVD-fmk (20 μM) and light (2 J/cm^2) (PDT+Z-DEVD-fmk).

protein into active subunits that were required to induce DNA fragmentation in isolated cell nuclei [6].

Wild type HL-60 cell lysates were prepared at 5, 15, 30 or 60 min following PDT. Immunoblot analysis showed that pro-caspase-3 cleavage was evident within 5 min after PDT (Fig. 2). Furthermore, virtually all DFF was processed by 15 min post-PDT, whereas PARP was not completely cleaved until 30 min post-PDT (Fig. 2). The earlier cleavage of DFF by caspase-3 may be attributable to the cytosolic localization of DFF and to the nuclear localization of PARP. Our observation that DFF was cleaved in PDT-treated cells undergoing apoptosis is consistent with that of Liu et al. [6] in which staurosporine was used as the pro-apoptotic stimulant in HeLa cells.

Treatment of the HL-60 cells with the caspase-3 inhibitor, Z-DEVD-fmk, prior to light activation of BPD-MA, prevented DFF and PARP cleavage (Fig. 3). The observation that Z-DEVD-fmk blocks PARP cleavage is consistent with results obtained by other investigators [2–4]. Our DFF results for HL-60 cells treated with PDT support evidence by Liu et al. suggesting that DFF is a substrate of caspase-3 [6]. Upon activation, it is believed that DFF may either translocate into the nucleus or interact with specific proteins on the nuclear envelope to trigger a signaling cascade resulting in endonuclease activation [6]. Further experiments are required in order to determine the mechanism of DFF-mediated endonuclease activation.

3.3. Overexpression of Bcl-X_L blocks PDT-induced caspase-3, PARP and DFF cleavage

To further examine upstream events regulating PDT-induced DFF activation, the role of the anti-apoptotic Bcl-X_L proto-oncogene was assessed. The status of Bcl-X_L, caspase-3, PARP and DFF in cytosolic extracts from PDT-treated cells was determined by Western immunoblotting. A minimal level of Bcl-X_L expression was detected in the HL-60/neo cells as compared to the HL-60/Bcl-X_L transfectants (Fig. 4). No apparent cleavage or size alteration of Bcl-X_L was observed in neither the PDT-treated HL-60/neo or HL-60/Bcl-X_L cells. Following light activation of BPD-MA, caspase-3, PARP and DFF were cleaved in the HL-60/neo cell lysates but not in the HL-60/Bcl-X_L cell extracts (Fig. 4). These results indicate that DFF activation by PDT is subject to Bcl-X_L regulation.

Previous studies have shown that Bcl-X_L localizes to the outer mitochondria membrane and prevents the activation of caspase-3 in response to a variety of apoptotic signals by blocking the release of cyt *c* from mitochondria [10]. HL-60 cells undergoing apoptosis exhibit an increase of cytosolic cyt *c* levels and a corresponding decrease of mitochondrial cyt *c*

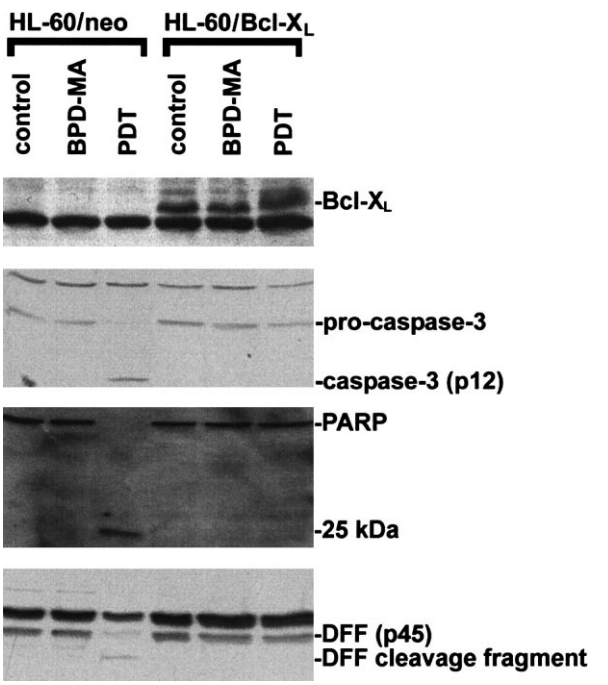

Fig. 4. Overexpression of Bcl-X_L in HL-60 cells blocks the cleavage of pro-caspase-3, PARP, and DFF induced by treatment with BPD-MA and light. Cells were lysed 1 h after light irradiation. Cell lysates were separated by SDS-PAGE followed by Western immunoblotting. Cells were treated with light (control), BPD-MA (100 ng/ml) or BPD-MA (100 ng/ml) plus light (2 J/cm^2) (PDT).

levels [21]. By regulating the release of cyt *c*, Bcl-X_L may impede apoptotic events by blocking caspase-3 activation and subsequent DFF activation which leads to endonuclease-mediated DNA fragmentation [21,22].

The findings in the present study demonstrate that PDT induces the activation of DFF through caspase-3 and that these processes are subject to Bcl-X_L regulation. It has previously been demonstrated that Bcl-X_L can bind to cytochrome c thereby blocking its release from the mitochondria [10] and that cyt *c* is necessary for caspase-3 activation [21–23]. Cyt *c* has also been shown to bind to Apaf-1 (apoptotic protease activating factor-1), a recently discovered human homolog of the nematode death-regulating CED-4 gene [23]. An interesting hypothesis is that cyt *c* may activate caspase-3 by disrupting the mitochondrial complex between Bcl-X_L and Apaf-1 [23]. Following the application of a pro-apoptotic stimuli such as PDT, cyt *c* may bind to Bcl-X_L thereby releasing Apaf-1 for caspase-3 processing. Thus, increasing Bcl-X_L may offset the effects of any increase in cyt *c* release by suppressing its binding capabilities to Apaf-1 thereby blocking further caspase-3 processing and DFF activation. The mechanisms by which DFF activates endonucleases involved in DNA fragmentation will be explored in future studies.

Acknowledgements: We thank Dr. Kapil Bhalla (Emory University School of Medicine, Atlanta, GA) for providing the HL-60/neo and HL-60/Bcl-X_L cells and Dr. Xiaodong Wang (University of Texas Southwestern Medical Center, Dallas, TX) for providing us with the DFF (p45) antibody. We are also grateful to Chris Carthy and Kathleen Watson (University of British Columbia) and Brent Rupnow (Stanford University) for critical review of the manuscript.

References

[1] Casciola-Rosen, L., Nicholson, D.W., Chong, T., Rowan, K., Thornberry, N.A., Miller, D. and Rosen, A. (1996) J. Exp. Med. 183, 1957–1964.
[2] Fernandes-Alnemri, T., Litwack, G. and Alnemri, E.S. (1994) J. Biol. Chem. 269, 30761–30764.
[3] Nicholson, W.D. et al. (1995) Nature 376, 37–43.
[4] Tewari, M. et al. (1995) Cell 81, 801–809.
[5] Wang, X., Zelenski, N.G., Yang, J., Sakai, J., Brown, M.S. and Goldstein, J.L. (1996) EMBO J. 15, 1012–1020.
[6] Liu, X., Zou, H., Slaughter, C. and Wang, X. (1997) Cell 89, 175–184.
[7] Boise, L.H. et al. (1993) Cell 74, 597–608.
[8] Minn, A.J., Velez, P., Schendel, S.L., Liang, H., Muchmore, S.W., Fesik, S.W., Fill, M. and Thompson, C.B. (1997) Nature 385, 353–357.
[9] Hsu, Y.T., Wolter, K.G. and Youle, R.J. (1997) Proc. Natl. Acad. Sci. USA 94, 3668–3672.
[10] Kharbanda, S. et al. (1997) Proc. Natl. Acad. Sci. USA 94, 6939–6942.
[11] Ibrado, A.M., Huang, Y., Fang, G., Liu, L. and Bhalla, K. (1996) Cancer Res. 56, 4743–4748.
[12] Chinnaiyan, A.M., Orth, K., O'Rourke, K., Duan, H., Poirier, G.G. and Dixit, V.M. (1996) J. Biol. Chem. 271, 4573–4576.
[13] Datta, R., Banach, D., Kojima, H., Talanian, R.V., Alnemri, E.S., Wong, W.W. and Kufe, D.W. (1996) Blood 88, 1936–1943.
[14] Gomer, C.J., Ferrario, A., Hayashi, N., Rucker, N., Szirth, B. and Murphree, A. (1988) Laser Surg. Med. 8, 450–463.
[15] Jamieson, C., Richter, A. and Levy, J. (1993) Exp. Hemat. 21, 629–634.
[16] Jamieson, C.H.M., McDonald, W.N. and Levy, J.G. (1990) Leukemia Res. 14, 209–219.
[17] Gluck, S., Chadderton, A. and Ho, A. (1996) Photochem. Photobiol. 63, 846–853.
[18] Granville, D.J., Levy, J.G. and Hunt, D.W. (1997) Cell Death Differ. 4, 623–629.
[19] Telford, W.G., King, L.E. and Fraker, P.J. (1994) J. Immunol. Methods 172, 1.
[20] Darzynkieicz, Z., Bruno, S., Del Bino, G., Gorczyca, W., Hotz, M., Lassota, P. and Traganos, F. (1992) Cytometry 13, 795–808.
[21] Yang, J. et al. (1997) Science 275, 1129–1132.
[22] Kluck, R.M., Bossy-Wetzel, E., Green, D.R. and Newmeyer, D.D. (1997) Science 275, 1132–1136.
[23] Zou, H., Henzel, W.J., Liu, X., Lutschg, A. and Wang, X. (1997) Cell 90, 405–413.