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Galectin-1 induces nuclear translocation of endonuclease G in caspase- and cytochrome *c*-independent T cell death

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Abstract

Galectin-1, a mammalian lectin expressed in many tissues, induces death of diverse cell types, including lymphocytes and tumor cells. The galectin-1 T cell death pathway is novel and distinct from other death pathways, including those initiated by Fas and corticosteroids. We have found that galectin-1 binding to human T cell lines triggered rapid translocation of endonuclease G from mitochondria to nuclei. However, endonuclease G nuclear translocation occurred without cytochrome *c* release from mitochondria, without nuclear translocation of apoptosis-inducing factor, and prior to loss of mitochondrial membrane potential. Galectin-1 treatment did not result in caspase activation, nor was death blocked by caspase inhibitors. However, galectin-1 cell death was inhibited by intracellular expression of galectin-3, and galectin-3 expression inhibited the eventual loss of mitochondrial membrane potential. Galectin-1-induced cell death proceeds via a caspase-independent pathway that involves a unique pattern of mitochondrial events, and different galectin family members can coordinately regulate susceptibility to cell death.

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Abbreviations: PS, phosphatidylserine; zVAD-fmk, z-Val-Ala-Asp(OMe)-CH_2F; zDEVD-fmk, z-Asp-Glu-Val-Asp(OMe)-CH_2F; PARP, poly(ADP-ribose)polymerase; 7AAD, 7-amino-actinomycin D; zDEVD-AFC, z-Asp-Glu-Val-Asp-7-amino-4-trifluoromethylcoumarin; $\Delta\psi m$, mitochondrial membrane potential; EndoG, endonuclease G; NAO, 10-*N*-nonyl acridine orange; AIF, apoptosis-inducing factor; tBid, truncated Bid; PI, propidium iodide; FITC, fluorescein isothiocyanate

Introduction

Cell death is crucial for proper development of multicellular organisms, for maintenance of immune homeostasis and for prevention of neoplastic disease. Death in many cell types is a complex process, utilizing multiple death signals and several parallel death pathways.[1–3] The complexity of cell death pathways reflects a requirement for tight control of the death process, while redundancy is ensured by overlapping cell death inducers and effectors.

Regulation of cell death is a conserved function of the galectins, a family of 14 mammalian lectins that are expressed in a wide variety of tissues. Galectins-1, -7, -8, -9 and -12 induce death of various cell types[4–6] including lymphocytes, keratinocytes, eosinophils, adipocytes and tumor cells, while intracellular galectin-3 expression protects T cells and some types of carcinoma cells from death.[6–10] We have demonstrated that galectin-1 can induce cell death of T cells and thymocytes, while other groups have shown that galectin-1 can kill B cells, breast cancer cells and prostate cancer cells.[11–13] The mechanism of galectin-1 induced T cell death is distinct from that mediated by Fas or glucocorticoids.[4,5] However, the galectin-1 death pathway in T cells is still poorly understood. Intriguingly, galectins are an evolutionarily ancient family of molecules, with homologs found in primitive organisms including multicellular fungi and sponges.[14] If cell death regulation is a conserved function of galectins in lower organisms, then components of the galectin-1 mediated T cell death pathway may have features common to the simpler cell death mechanisms of organisms such as fungi.

We have found that the galectin-1 death pathway in T cells has several novel features. While galectin-1-mediated T cell death was caspase-independent, galectin-1 binding to T cells results in translocation of endonuclease G (EndoG)[15,16] to the nucleus without release of other mitochondrial death effectors. In addition, intracellular galectin-3 inhibited galectin-1 induced T cell death, indicating that different galectin family members can coordinately regulate cell fate.

Results

Galectin-1 induced cell death is caspase independent

Galectin-1 induced cell death has many features such as rapid phosphatidylserine (PS) externalization, membrane blebbing and nuclear DNA fragmentation, which can be mediated by caspase proteases. However, galectin-1 induced T cell death appeared to be caspase-independent.

We examined the effects of caspase inhibitors on galectin-1 induced death of CEM and MOLT-4 T cells. Fas induced death of CEM T cells was used as a positive control, as MOLT-4 cells are resistant to Fas killing.[17] Cells were treated with either z-Val-Ala-Asp(OMe)-CH_2F (zVAD-fmk), a general caspase inhibitor, or z-Asp-Glu-Val-Asp(OMe)-CH_2F (zDEVD-fmk), a downstream caspase inhibitor, for 1 h prior to the addition of galectin-1, and death assessed by annexin V binding. As shown in Figure 1a, both zVAD-fmk and zDEVD-fmk significantly inhibited Fas induced CEM cell death. However, 100 μM zVAD-fmk and zDEVD-fmk had no detectable effect on galectin-1 induced death of either CEM or MOLT-4 cells. As PS externalization can occur independently of caspase activity,[2] we also asked whether markers of cell death downstream of PS externalization required caspase activity, by examining the effects of caspase inhibitors on cell loss and membrane permeability to 7-amino-actinomycin D (7AAD). zVAD-fmk and zDEVD-fmk also did not inhibit galectin-1 induced cell loss or uptake of 7AAD, while these inhibitors did block cell loss and 7AAD uptake in Fas induced cell death (Figure 1b).

As caspase inhibitors may not completely inhibit all caspase activity, caspase activation was also assayed using the endogenous caspase substrate poly(ADP-ribose)polymerase (PARP), an 116 kDa protein that can be cleaved by caspases to an 85 kDa form during apoptosis. The 85 kDa PARP cleavage fragment appeared in anti-Fas mAb treated cells (Figure 1c lane 4). However, galectin-1 treated CEM and MOLT-4 (Figure 1c lanes 2 and 6) cells did not contain increased amounts of the 85 kDa PARP cleavage product compared to controls (Figure 1c lanes 1 and 5), although galectin-1 and anti-Fas treatment resulted in equivalent levels of cell loss. We also examined cleavage of DFF40/CAD, another endogenous caspase substrate,[18] after galectin-1 treatment, but detected no DFF40/CAD cleavage after galectin-1 treatment (data not shown).

Cleavage of zDEVD-7-amino-4-trifluoromethylcoumarin (zDEVD-AFC), a synthetic fluorogenic caspase substrate, was also measured. Extracts of CEM cells treated with anti-Fas mAb cleaved zDEVD-AFC, resulting in the release of free AFC (Figure 1d), and zDEVD-AFC cleavage in Fas-treated cells was inhibited by zDEVD-fmk (data not shown). However, we detected no cleavage of zDEVD-AFC by extracts of galectin-1 treated CEM and MOLT-4 cells (Figure 1d).

Finally, we directly examined activation of several pro-caspases, including 2, 3, 4, 6, 8, 9 and 10, to active forms, by immunoblotting with a panel of specific antibodies. Although we detected activation of pro-caspases in anti-Fas mAb treated CEM cells, we did not detect activation of any of these pro-caspases in galectin-1 treated CEM and MOLT-4 cells (data not shown). Therefore, galectin-1 induced cell death appears to operate via a caspase-independent mechanism.

As non-caspase proteases have also been implicated in cell death,[2,19–22] we also examined the ability of several other protease inhibitors to inhibit galectin-1 cell death. We observed no reduction in galectin-1 induced PS exposure with calpain inhibitors I and II, serine protease inhibitors TPCK and TLCK, cathepsin inhibitors pepstatin and CA-074 Me, or the proteasome inhibitor lactacystin (data not shown).

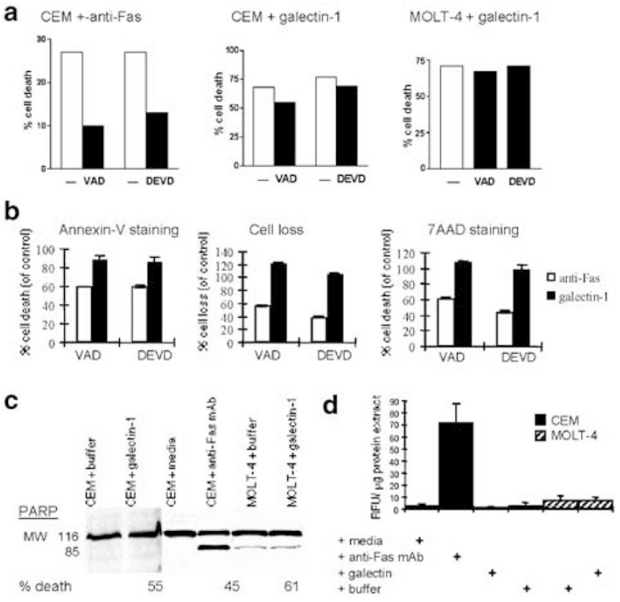


Figure 1 Galectin-1 induced T cell death is caspase-independent. (**a**) zVAD-fmk and zDEVD-fmk did not inhibit galectin-1 induced PS externalization. Cells were pretreated with 100 μM zVAD-fmk or zDEVD-fmk or buffer control (—) for 1 h prior to addition of anti-Fas mAb or control IgM (left, CEM cells), or galectin-1 or buffer control (center, CEM cells; right, MOLT-4 cells), for 4 h. Cell death was determined by staining with annexin V/PI as described in Methods. Values are mean of triplicate determinations from one of three replicate experiments. (**b**) zVAD-fmk and zDEVD-fmk did not block appearance of downstream hallmarks of galectin-1 cell death. CEM cells were treated with 100 μM of indicated inhibitors as described above prior to addition of anti-Fas mAb, galectin-1, or appropriate controls for 6 h. Cell death measured by annexin V (left panel) or 7-AAD uptake (right panel), or cell loss (center panel) was expressed as percent of control T cell death in the absence of the inhibitors. (**c**) PARP, an endogenous caspase substrate, was not cleaved during galectin-1 mediated cell death. In total, 50 μg of total cell extracts from CEM and MOLT-4 cells, treated as indicated with galectin-1, anti-Fas mAb, or appropriate controls, were loaded in each lane. PARP cleavage fragments were detected by immunoblotting. The 85 kDa PARP cleavage fragment was detected only in cells treated with anti-Fas mAb. Results are representative of three separate experiments. (**d**) zDEVD-AFC, a synthetic caspase substrate, was not cleaved after galectin-1 addition. Total cell extracts of CEM and MOLT-4 cells treated as indicated with galectin-1, anti-Fas mAb, or appropriate controls were incubated with zDEVD-AFC. Free fluorescence was detected on a Cytofluor plate reader and reported as relative fluorescence units (RFU). zDEVD-AFC cleavage was only detected in samples with extracts of anti-Fas treated cells. Results are representative of three separate experiments

Galectin-1 cell death induces a novel pattern of mitochondrial events

Mitochondrial events that occur during different types of cell death include loss of mitochondrial membrane potential ($\Delta\psi$m), release of cytochrome *c* to the cytosol, and translocation of apoptosis-inducing factor (AIF) and EndoG to the nucleus.[15,16,22–27] Suprisingly, we observed a novel pattern of mitochondrial events during galectin-1 mediated cell death. Galectin-1 treatment induced rapid EndoG translocation from mitochondria to nuclei without cytochrome *c* release, while loss of $\Delta\psi$m was only detected after EndoG translocation.

We examined the $\Delta\psi$m of CEM cells undergoing galectin-1 and anti-Fas mAb induced death. Figure 2a shows that $\Delta\psi$m was maintained in both galectin-1 and anti-Fas mAb treated cells after 1 h. However, at this time, significant PS externalization had occurred in galectin-1 treated cells (Figure 2a). At

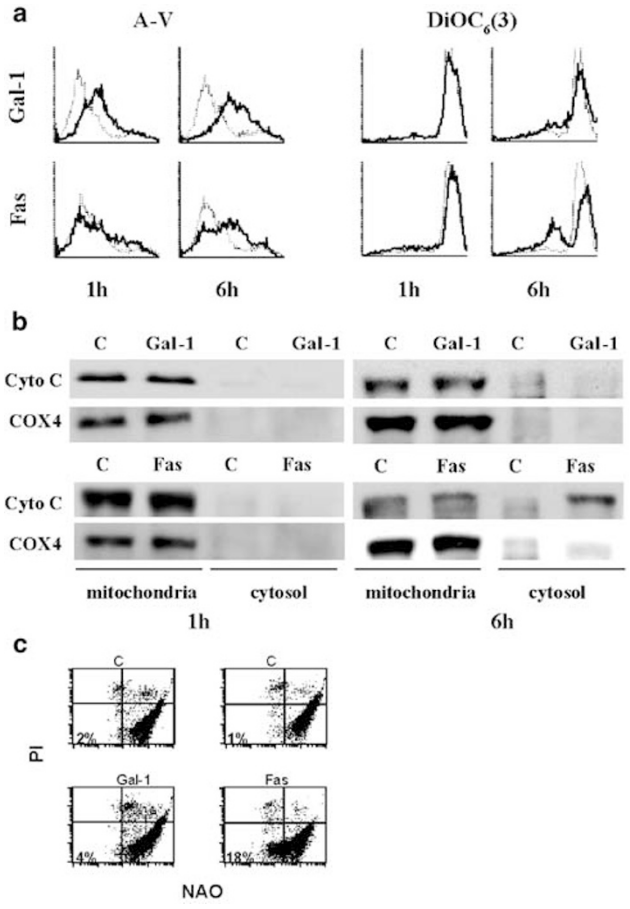


Figure 2 Mitochondrial events in galectin-1 cell death. (**a**) Loss of mitochondrial membrane potential. CEM T cells were treated with galectin-1 (solid line) or buffer control (dotted line) (top panel), or with anti-Fas mAb (solid line) or control IgM (dotted line) (bottom panel) for times indicated, and PS externalization was determined by annexin V binding (A-V, left). Treated cells were also stained with $DiOC_6(3)$ to detect loss of $\Delta\Psi m$ (right). Results are representative of three or more separate experiments. (**b**) Cytochrome *c* was not released to the cytosol during galectin-1 mediated cell death. CEM cells were treated with galectin-1 (Gal-1), buffer control (C), anti-Fas mAb, or IgM control (C) for times indicated; at 6 h, galectin-1 treated cells demonstrated 34% cell death, and Fas treated cells demonstrated 42% cell death. Mitochondrial and cytosolic fractions were probed with antibodies to cytochrome *c* and COX4, a mitochondrial marker. Cytosolic cytochrome *c* was detected in anti-Fas mAb treated CEM cells, but was not detected in galectin-1 or control treated CEM cells up to 6 h after galectin-1 addition. Results are representative of three separate experiments. (**c**) Mitochondrial cardiolipin was not reduced during galectin-1 death. CEM cells were treated for 3 h with galectin-1 or anti-Fas mAb, or appropriate controls, and NAO uptake was measured. The percent of NAO^{low}, PI^- cells, indicating loss of mitochondrial cardiolipin, is shown in the lower left quadrant

6 h, loss of $\Delta\psi m$ was detected in cells undergoing both galectin-1 and Fas induced death. However, at 6 h, a smaller fraction of galectin-1 treated cells were $DiOC_6(3)^{low}$ than were annexin V^+. These results indicate that loss of $\Delta\psi m$ is not an early step in galectin-1 induced cell death.

While $\Delta\psi m$ was maintained during the early stages of galectin-1 death, other mitochondrial events such as release of cytochrome *c* can trigger apoptosis without loss of $\Delta\psi m$.[26] To examine cytochrome *c* release, CEM cells were treated with galectin-1 or anti-Fas mAb and the translocation of cytochrome *c* to the cytosol was detected by immunoblotting. Annexin V/PI staining demonstrated 42% cell death of Fas treated cells and 34% cell death of galectin-1 treated cells in these samples. Treatment of cells with anti-Fas mAb resulted in release of cytochrome *c* to the cytosol. In contrast, we detected no release of cytochrome *c* in the cytosol of galectin-1 treated cells by 6 h, although the amount of cell death induced by galectin-1 and Fas were comparable (Figure 2b). Thus, galectin-1-mediated cell death does not appear to require the release of cytochrome *c* from the mitochondria to the cytosol.

Loss of cardiolipin from mitochondria is associated with release of cytochrome *c* associated with the inner mitochondrial membrane during apoptosis.[27] We examined loss of mitochondrial cardiolipin in galectin-1 and Fas-treated cells using the cardiolipin-specific dye 10-*N*-nonyl acridine orange (NAO); a decrease in mitochondrial cardiolipin results in decreased cell staining with NAO.[28] Cardiolipin loss was detected after 3 h in Fas-treated cells, while no decrease in NAO staining was detected in galectin-1 treated cells (Figure 2c), consistent with the lack of cytochrome *c* release in galectin-1 treated cells seen in Figure 2b.

DNA degradation has been demonstrated in galectin-1-mediated T cell death by several methods, including TUNEL labeling, detection of cells with subdiploid amounts of DNA and DNA laddering.[4,5,29] However, as mentioned above, galectin-1 induced cell death does not appear to involve the caspase-dependent endonuclease DFF40/CAD. EndoG has been identified as a mitochondrial endonuclease responsible for nucleosomal DNA fragmentation.[15,16,24] In mouse embryonic fibroblast (MEF) cells triggered to die by ultraviolet irradiation or treatment with TNF and cycloheximide, EndoG translocated from mitochondria to nuclei and cleaved nuclear DNA; both EndoG translocation and nuclease activity were caspase-independent.[15]

We examined the subcellular location of EndoG at several time points after the initiation of galectin-1-mediated death of CEM T cells (Figure 3). Strikingly, translocation of EndoG from the mitochondria to the nucleus occurred very rapidly in galectin-1 induced T cell death. Figure 3a demonstrates the appearance of EndoG in nuclear fractions from galectin-1 treated cells, while there was no increase in another mitochondrial protein, AIF, in these nuclear fractions. By immunofluorescence analysis, nuclear EndoG was detected in a significant fraction of the cells by 1 h after addition of galectin-1 (Figure 3c), although no detectable increase in nuclear EndoG occurred at earlier timepoints. By 6 h after galectin-1 addition, the majority of the cells had translocated EndoG to the nucleus (Figure 3c). The rapid nuclear translocation of EndoG in galectin-1 treated cells occurred in the absence of significant loss of mitochondrial membrane potential and without cytochrome *c* release (Figure 2).

Figure 4 demonstrates the subcellular localization of EndoG in CEM cells after galectin-1 treatment. In control-treated cells, cytochrome *c* and EndoG remained co-localized in punctate cytoplasmic structures that ring the nucleus, demonstrating the mitochondrial localization of EndoG. However, in CEM cells treated with galectin-1, EndoG was detected in the nucleus (top panel), while cytochrome *c* remained in punctate structures in the cytoplasm, consistent with results in Figure 2 demonstrating lack of cytochrome *c*

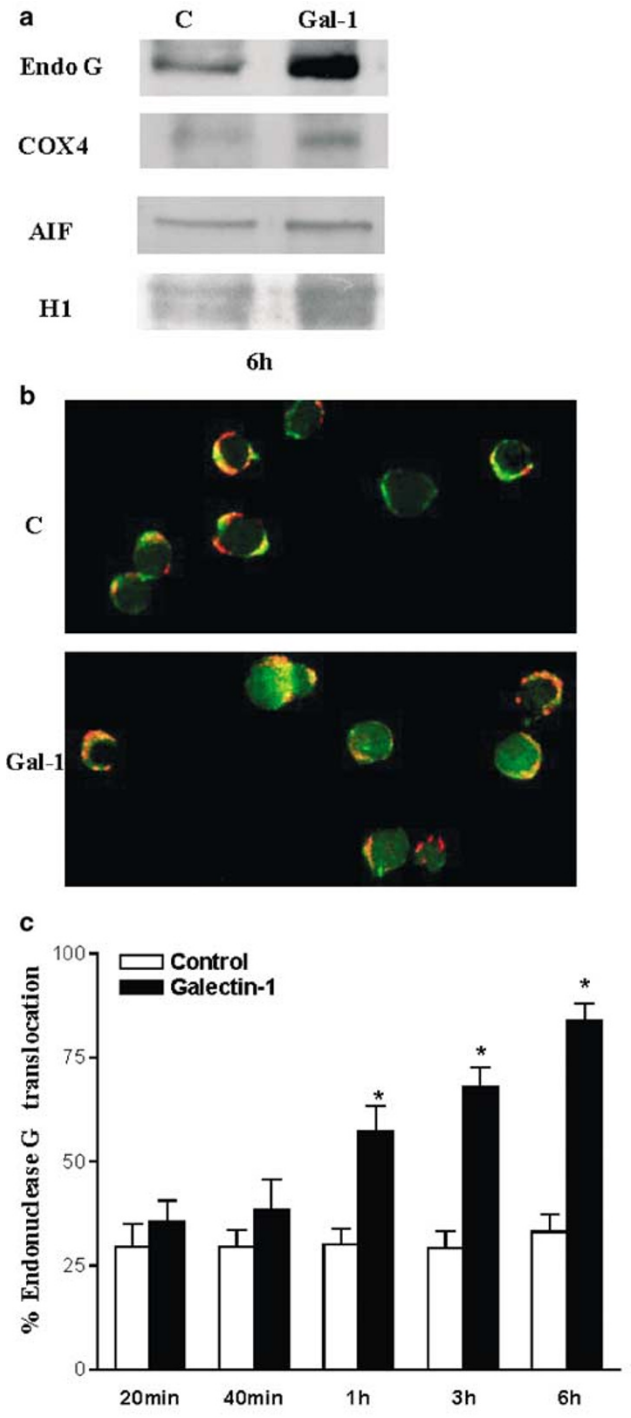


Figure 3 EndoG translocation to the nucleus occurs rapidly in galectin-1 induced death. (**a**) CEM cells were treated with galectin-1 or buffer control for 6 h. Cells were fractionated, and the nuclear fraction was separated by SDS-PAGE and transferred to nitrocellulose. The membrane was sequentially probed with antibody to EndoG, AIF, COX4 as a control for mitochondrial contamination, or histone H1 as a nuclear loading control. (**b**) CEM cells were treated with galectin-1 or buffer control for various times up to 6 h. EndoG (green) and cytochrome *c* (red) were detected by immunofluorescence, and nuclear translocation of EndoG at 6 h is shown. (**c**) Quantification of labeled nuclei from B at the indicated time points. At least 50 cells in five microscopic fields were counted for each time point, and the % cells with nuclear localization of EndoG determined. Data are mean $\pm$ S.E.M. for three determinations. *$P<0.05$ *versus* control

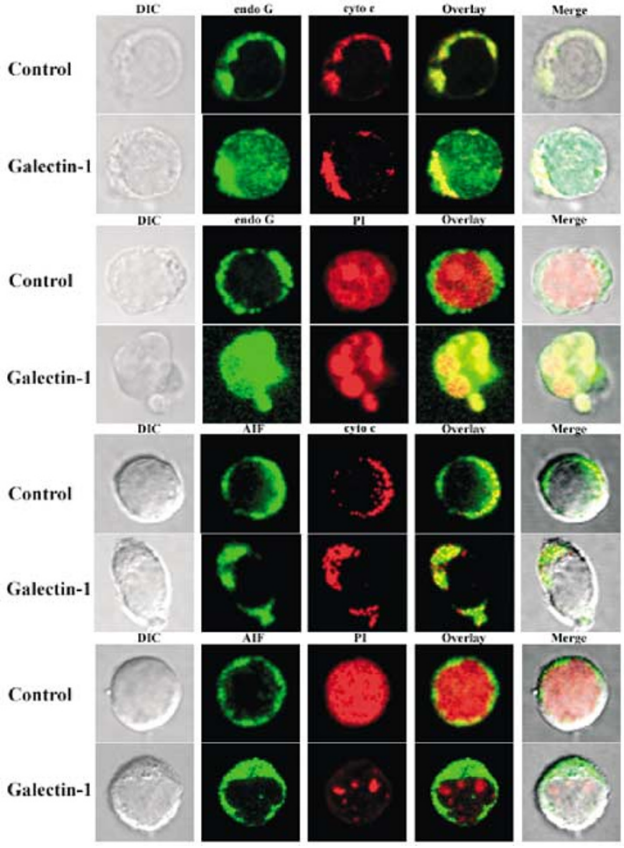
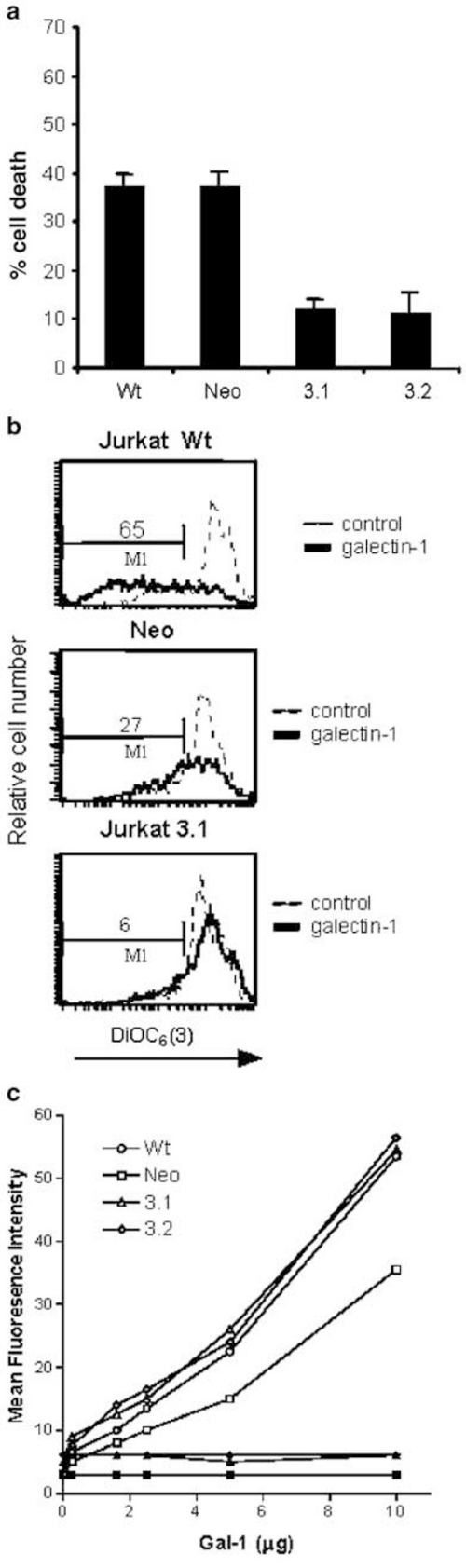


Figure 4 EndoG, but not AIF, translocates to the nucleus during galectin-1 induced cell death. In each panel (two rows per panel), cells were treated with buffer control or galectin-1 for 3 h. (Top panel) Subcellular localization of EndoG (green) and cytochrome *c* (red) detected with rabbit anti-human EndoG and mouse anti-human cytochrome *c*. In control cells, EndoG and cytochrome *c* co-localize (yellow) in punctate structures in the cytoplasm, indicating mitochondrial localization. In galectin-1 treated cells, EndoG staining of nuclei is apparent, while cytochrome *c* staining remains punctate in the cytoplasm. (Second panel) Cells treated as above were stained with anti-EndoG (green) and PI (red). In control treated cells, punctate EndoG staining is present in the cytoplasm, while the nucleus stains with PI. In galectin-1 treated cells, EndoG is present in nuclear fragments that stain with both anti-EndoG and PI (yellow). (Third panel) Cells treated as above were stained with anti-AIF (green) and anti-cytochrome *c* (red). AIF and cytochrome *c* remain co-localized in galectin-1 treated cells. (Fourth panel) Cells treated as above were stained with anti-AIF (green) and PI (red). No nuclear translocation of AIF was observed in galectin-1 treated cells. As a positive control, CEM cells were treated with staurosporine or buffer control for 3 h and stained with anti-AIF and anti-cytochrome *c* or PI. AIF nuclear translocation was observed in 56% of staurosporine treated cells, but in $<5\%$ of buffer control treated cells (data not shown)

release in galectin-1 treated cells. In cells that had nuclear fragmentation, a sign of DNA degradation, EndoG was detected in the nuclear fragments (second panel).

As mentioned above, AIF is another mitochondrial protein involved in caspase-independent cell death triggered by a variety of agents, including staurosporine and dexamethasone.[30–33] We examined the subcellular localization of AIF after galectin-1 addition to CEM T cells. As shown in Figure 4, no increase in nuclear translocation of AIF was detected in galectin-1 treated cells compared to cells treated with buffer alone (third and fourth panels), confirming the biochemical results shown in Figure 3; nuclear staining for AIF was



detected in 6% of cells treated with buffer alone, and in 5% of cells treated with galectin-1. In contrast, staurosporine treated CEM cells were examined for AIF nuclear translocation as a positive control; staurosporine treatment resulted in nuclear translocation of AIF in 56% of the CEM cells (data not shown). Thus, nuclear translocation of EndoG occurs early in galectin-1 induced T cell death in the absence of cytochrome *c* release or AIF nuclear translocation from the mitochondria, indicating that release of various mitochondrial effectors can be uncoupled in different death pathways,[34] although the role of EndoG in galectin-1 induced cell death remains unknown.

Galectin-3 blocks galectin-1 mediated cell death

Galectin-3 is the only known galectin with antiapoptotic activity. Intracellular galectin-3 expression inhibited death of T cells, myeloid cells and breast cancer cells induced by a variety of triggers such as Fas, nitric oxide, staurosporine, cisplatin and detachment of adherent cells.[6–10,35–37] However, little is known about the ability of different pro- and anti-death galectins to coordinately regulate cell death. We examined Jurkat T cells transfected with galectin-3 or vector alone for susceptibility to galectin-1. Galectin-3 expression in these cells has been shown to inhibit death induced by anti-Fas or staurosporine.[7] We confirmed that, as previously shown,[7] the two galectin-3 expressing clones 3.1 and 3.2 produce abundant intracellular galectin-3, while no galectin-3 is detectable on the cell surface (data not shown). Figure 5a demonstrates that the Jurkat 3.1 and 3.2 cells were resistant to galectin-1 induced cell death, compared to the parental (wt) and control transfected (neo) cells. Thus, intracellular galectin-3 blocked cell death triggered by extracellular galectin-1.

The antiapoptotic effect of galectin-3 has been attributed to the ability of galectin-3 to block mitochondrial events in cell death.[7,8,10] We examined the mitochondrial membrane potential of Jurkat wt, neo and 3.1 cells treated with galectin-1 for 6 h. As shown in Figure 5b, intracellular galectin-3 expression also blocked galectin-1 induced disruption of $\Delta\psi$m. To confirm that the loss of susceptibility to galectin-1 in galectin-3 transfected cells did not result from decreased availability of galectin-1 receptors on the cell surface, we performed binding assays using biotinylated galectin-1 to measure the relative binding of galectin-1 to the

Figure 5 Intracellular galectin-3 inhibits galectin-1 mediated cell death. (**a**) Jurkat T cells (wt), transfected with vector control (neo) or with galectin-3 (clone 3.1 and 3.2), were treated with galectin-1 or buffer control for 6 h and cell death was determined as described in Methods. Results are representative of three or more separate experiments. (**b**) Galectin-3 expression inhibited galectin-1 mediated loss of $\Delta\psi$m. During cell death assays performed above, cells were stained with $DiOC_6(3)$ to measure $\Delta\Psi$m. In cells expressing galectin-3, galectin-1 caused no loss of $\Delta\Psi$m, while wild type or neo Jurkat T cells demonstrated loss of $\Delta\Psi$m after addition of galectin-1. (**c**) Galectin-3 transfectants bind galectin-1 in a carbohydrate dependent manner. The indicated amount of biotinylated galectin-1 was added to wildtype, neo, or galectin-3 transfected Jurkat cells (clones 3.1 and 3.2) for 1 h at 4°C. Bound galectin-1 was detected with avidin-FITC and binding quantitated by flow cytometry. Open symbols indicate galectin-1 binding to the relevant cell line in the absence of lactose; closed symbols indicate galectin-1 binding to the relevant cell line in the presence of 100 mM lactose

cells. As shown in Figure 5c, expression of galectin-3 did not reduce galectin-1 binding to the surface of the cells, compared with the parental cells or the vector control. In addition, binding to all cell lines was completely abrogated in the presence of 100 mM lactose, demonstrating that all galectin-1 binding we observed was carbohydrate-dependent.

Galectin-1 induced cell death does not require *de novo* protein synthesis

Galectin-1 induced PS exposure on both T cell lines and thymocytes occurs rapidly, with changes in membrane asymmetry detectable after only 20 min exposure to galectin-1,[38] implying that galectin-1 induced cell death does not require *de novo* protein synthesis. To determine the requirement for protein synthesis in galectin-1 cell death, we examined the effect of cycloheximide; as seen in Figure 6, incubation with cycloheximide did not block galectin-1 induced PS externalization. Furthermore, pretreatment with cycloheximide did not inhibit galectin-1 induced cell loss, although this concentration of cycloheximide was effective in inhibiting 98% of dexamethasone induced cell death (data not shown). Thus, *de novo* protein synthesis is not an absolute requirement for galectin-1 cell death.

Reactive oxygen species can also participate in cell death. We measured peroxides and superoxides after galectin-1 treatment of CEM and MOLT-4 cells, but no increase in reactive oxygen species was detected in galectin-1 treated cells (data not shown). Also, preincubation with the antioxidants ascorbate and *N*-acetylcysteine did not protect cells from galectin-1 death, indicating that this pathway does not require generation of reactive oxygen species.

We also examined ceramide generation and calcium flux as possible early events in galectin-1 death. Ceramide generation can initiate caspase-independent cell death.[39,40] However, we did not detect significant ceramide generation during galectin-1 triggered killing, and inhibitors of ceramide synthase and acidic sphingomyelinase did not block galectin-1 cell death (data not shown). Finally, calcium flux has been reported to regulate externalization of PS during apoptosis.[41] Addition of galectin-1 to CEM and MOLT-4 cells induced an increase in intracellular calcium; while addition of EGTA blocked the galectin-1 mediated calcium flux, it did not significantly inhibit galectin-1 triggered PS externalization (data not shown). This is consistent with our earlier finding that galectin-1 could induce T cell death in the absence of calcium flux.[42] Thus, increases in intracellular ROS, ceramide or calcium do not appear to be essential for galectin-1 cell death.

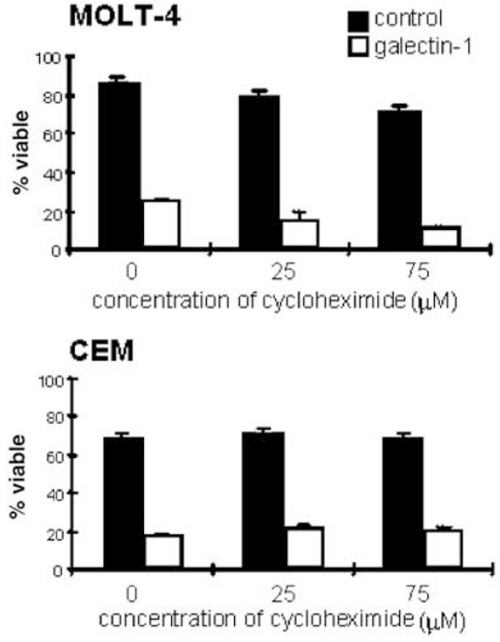


Figure 6 *De novo* protein synthesis is not required for galectin-1 induced cell death. MOLT-4 cells were treated with the indicated concentrations of cycloheximide and 20 μM galectin-1 or buffer control for 12 h. CEM cells were treated identically, but incubated with galectin-1 or buffer control for only 4 h to reduce background death due to cycloheximide. % viable cells, analyzed by annexin V and PI uptake, was determined as described in Methods. Values are mean $\pm$ S.E.M. of three separate experiments conducted in triplicate

Discussion

Galectin-1, an endogenous lectin expressed in many different tissues, can induce cell death of T and B cells, thymocytes, breast cancer cell lines, and prostate cancer cell lines.[6] During galectin-1 triggered death, T cells demonstrate PS externalization, chromatin condensation and margination, DNA fragmentation and membrane blebbing. While galectin-1 mediated cell death is distinct from death pathways triggered by Fas or glucocorticoids,[4,5,38] the mechanism of galectin-1 mediated T cell death is not well understood. Our studies demonstrate that galectin-1 induced T cell death is caspase-independent, involves rapid nuclear translocation of EndoG from mitochondria without detectable cytochrome *c* release or AIF translocation and prior to the loss of $\Delta\psi$m, and does not require *de novo* protein synthesis.

EndoG is a mitochondrial protein that is an important apoptogenic endonuclease.[15,16] During MEF cell death induced by UV radiation or by treatment with TNF plus cycloheximide, EndoG translocates from mitochondria to the nucleus and cleaves nuclear DNA in a caspase-independent manner.[15] As shown in Figures 1, 3 and 4, galectin-1-mediated cell death involved rapid EndoG translocation in the absence of caspase activation. In cells treated with galectin-1, EndoG translocation occurred in most cells prior to the loss of $\Delta\psi$m and without detectable release of cytochrome *c* or AIF (Figures 2, 4). This is in contrast to MEF cell death induced by UV radiation and TNF, and Fas mediated death of lymphoid cells, in which both EndoG and cytochrome *c* are released from mitochondria.[15,43] While cytochrome *c* release can occur without disruption of $\Delta\psi$m or mitochondrial function,[26,44] the absence of detectable cytochrome *c* release during EndoG translocation is a novel feature of galectin-1 T cell death. As mitochondrial remodeling has been shown to be important for cytochrome *c* release, our results suggest that galectin-1 binding does not result in the same mitochondrial structural alterations that are caused by other apoptotic triggers,[45] as indicated by the lack of cytochrome *c* release observed in galectin-1 treated cells (Figure 2b).

While several studies have shown concomitant release of EndoG and AIF during cell death,[33,34,46] EndoG alone was sufficient for DNA degradation in MEF cells.[15] Thus, the lack of AIF release that we observed may indicate that EndoG participates alone in galectin-1 mediated cell death, or may indicate that other factors cooperate with EndoG in the galectin-1 death pathway, although the precise role for EndoG in galectin-1 cell death has not been determined. Though the exact mechanisms regulating release and translocation of specific mitochondrial proteins during cell death are not completely understood, release appears to be regulated and to be protein specific, rather than the result of general mitochondrial destruction; proposed models include a hierarchical model of mitochondrial protein release with different thresholds for release of specific proteins, as well as selective channels for different mitochondrial effectors.[25,33,46–48] For example, in MEF cells treated with UV radiation or TNF, translocation of EndoG did not result in release of mitochondrial hsp70.[15] The initiator(s) of EndoG release from mitochondria during galectin-1 induced death are not known; while truncated Bid (tBid) can trigger EndoG release from mitochondria in Fas-induced liver cell death,[43] generation of tBid results from caspase cleavage, and tBid also releases cytochrome *c*,[43,44,49,50] features that we did not observe in galectin-1 treated cells. The roles of tBid and other Bcl family members in galectin-1 death remain to be elucidated.

Several members of the galectin family can positively regulate cell death, while the only antiapoptotic galectin is galectin-3. As mentioned above, intracellular galectin-3 can block cell death induced by a variety of stimuli, including staurosporine, nitric oxide, cisplatin, Fas ligation and death triggered by loss of adhesion.[7,9,10,37] We found that galectin-3 expression in Jurkat T cells also blocked galectin-1 induced cell death. Galectin-3 expression in these clones was entirely intracellular, as no galectin-3 was detected on the cell surface. In addition, galectin-1 bound to all the Jurkat cell lines in a carbohydrate-dependent manner, as detected by flow cytometry, regardless of galectin-3 expression status (Figure 5c). Therefore, the protective effect of galectin-3 was not due to simple competitive inhibition with galectin-1 binding for cell surface carbohydrate ligands. In our study, intracellular expression of galectin-3 inhibited the eventual loss of mitochondrial membrane potential in galectin-1 treated cells. While intracellular galectin-3 has been shown to prevent release of cytochrome *c* in breast cancer cells treated with cisplatin or staurosporine,[10] this specific event is likely not contributing to resistance to galectin-1 death, as we detected no release of cytochrome *c* in galectin-1 treated T cells (Figure 2). However, galectin-3 expression may directly stabilize mitochondria and prevent other mitochondrial changes in galectin-1 death. Alternatively, galectin-3 can bind to antiapoptotic Bcl-2,[7,8] and may antagonize the decrease in Bcl-2 that has been observed in galectin-1 induced T cell death.[51]

We have previously shown that galectin-1 induced T cell death proceeds rapidly, suggesting that *de novo* protein synthesis was not required for death.[38] This was confirmed in the present study, as cycloheximide treatment did not inhibit galectin-1 induced T cell death; similarly, protein synthesis was not required for EndoG translocation or death of MEF cells.[15] In contrast, Rabinovich *et al* found activation of the AP-1 transcription factor during galectin-1 apoptosis in rat T cells,[29] and also described caspase activation triggered by galectin-1 binding.[51] It is possible that galectin-1 may be activating different death pathways in different cell types. Alternatively, galectin-1 may activate more than one cell death mechanism, similar to the dual death mechanisms activated by Fas.[3] Of note, galectin-9 has been shown to trigger death of various cell types via a caspase-1 dependent pathway.[52] In addition, Cummings and co-workers[53] have determined that galectin-1 binding to some cell types does not result in DNA cleavage, but that the galectin-1 induced PS exposure on the cell membrane is sufficient for phagocytosis of the cells by macrophages. Thus, galectin-1 binding may result in different endpoints in different cell types.

Galectin-1 induced cell death may utilize a death mechanism that has been conserved during evolution. Galectins are evolutionarily ancient molecules, with homologs found in primitive organisms including multicellular fungi, sponges and *C. elegans*.[14] The galactose-containing ligands preferentially recognized by galectins are present on cell surface glycoconjugates of these primitive organisms.[54] Mitochondrial EndoG release participates in apoptosis in *C. elegans*, with no requirement for cytochrome *c* release.[16,33] Intriguingly, aspects of galectin-1 induced cell death, such as caspase- and cytochrome *c*-independence, are reminiscent of cell death pathways of organisms such as yeast and *Dictyostelium*.[55,56]

Understanding the unique pathway of galectin-1 induced cell death is critical for development of new approaches to regulating cell survival. The potential of galectin-1 in modulating immune responses in T cell-dependent autoimmune disorders and in cancer has been demonstrated in animal models.[6,57] In addition, galectin-1 may also be a useful antineoplastic agent, by killing cells that have escaped other apoptotic triggers.[12,13] That the galectin-1 death pathway is caspase-independent and appears to be distinct from other death pathways suggests that galectin-1 can synergize with other apoptotic agents, as has been shown with T cell receptor engagement or dexamethasone treatment.[5,58] Further elucidation of the galectin-1 death mechanism will facilitate identification of target cells susceptible to this type of death, and the design of agents to therapeutically manipulate this novel death pathway.

Materials and Methods

Cell lines and reagents

MOLT-4 human T lymphoblastoid cells were purchased from ATCC (Rockville, MD, USA). CEM T cells were the gift of Dr. Blair Ardman, New England Medical Center, Boston, MA, USA. Jurkat T cells were obtained and galectin-3 expressed and purified as previously described.[7] Reagents were from the indicated suppliers: cycloheximide (ICN, Costa Mesa, CA, USA), z-Val-Ala-Asp(OMe)-CH_2F (zVAD-fmk) and z-Asp-Glu-Val-Asp(OMe)-CH_2F (zDEVD-fmk) (Enzyme Systems Products, Livermore, CA, USA), zDEVD-7-amino-4-trifluoromethylcoumarin (zDEVD-AFC) (Kamiya, Seattle, WA, USA), $DiOC_6(3)$, 10-*N*-nonyl acridine orange (NAO) and Prolong Antifade Mounting Media (Molecular Probes, Eugene, OR, USA), annexin V-FITC Apoptosis kit (R&D Systems, Minneapolis,

MN, USA), pepstatin (Roche, Indianapolis, IN, USA), CA-074 Me (Calbiochem, San Diego, CA, USA). Recombinant galectin-1 was synthesized as previously described.[4] Antibodies were from the following sources: mouse anti-human Fas, clone CH11 and mouse anti-human histone H1, clone AE-4 (Upstate Biotechnology, Lake Placid, NY); anti-poly(ADP-ribose)polymerase (PARP), clone C2.10 (Enzyme Systems Products, Livermore, CA, USA); anti-cytochrome *c*, clone 7H8.2C12 (Pharmingen, San Diego, CA, USA); anti-AIF (ProSci Inc., Poway, CA, USA); fluorescein isothiocyanate (FITC) conjugated goat-anti rabbit serum (Jackson Laboratories, West Grove, PA, USA); Texas Red conjugated goat anti-mouse IgG (Southern Biotech Associates, Birmingham, AL, USA). Rabbit anti-EndoG was produced as in Li *et al*.[15]

Galectin-1 and Fas death assays

Galectin-1 death assays were performed as described (4) with indicated times. For Fas death assays, 2×10^6 cells in 400 μl final volume were treated with 1 μg/ml anti-Fas mAb and incubated at 37°C for indicated times. Analysis was performed on a BectonDickinson FACScan flow cytometer using CellQuest software. Cell loss was determined by forward v. side scatter gating, as described.[5] Cells were analyzed for annexin V binding and uptake of propidium iodide (PI) or 7-amino-actinomycin D (7AAD). Percent viable cells was calculated as % viable cells = [100 × (# annexin V^-, PI^- cells)/(total number of cells)], and percent cell death was calculated as: % cell death = 100 × [1 − % viable cells (galectin-1 or Fas)/% viable (control)].

Galectin-1 binding

Binding of biotinylated galectin-1 was determined by flow cytometric analysis exactly as described in Amano *et al*[59] in the presence or absence of 100 mM lactose to demonstrate carbohydrate-specific binding.

Immunoblotting

For PARP analysis, 50 μg of total cell extract was separated by 12% SDS-PAGE and immunoblotting was performed according to the manufacturer's protocol. For cytochrome *c* and EndoG analysis, mitochondria were extracted from whole-cell lysates using the ApoAlert Cell Fractionation kit (BD Biosciences, Palo Alto, CA, USA), according to the manufacturer's directions. Briefly, cells were homogenized in ice cold mitochondrial fractionation buffer using a glass Dounce homogenizer and Teflon pestle. Cell homogenates were centrifuged at 750 × *g* for 10 min to remove cell nuclei. The mitochondrial fraction was obtained by centrifugation at 10 000 × *g* for 25 min at 4°C and the supernatant was used as cellular cytosolic extract. In total, 30 μg of each fraction was separated on 15% SDS-PAGE and immunoblotting was performed according to the manufacturer's protocol. Antibodies to cytochrome *c* and the mitochondrial control cytochrome oxidase 4 (COX4) were included in the kit. Blots were visualized using Enhanced Chemiluminescence (ECL) (Amersham, Arlington Heights, IL, USA).

DEVD-AFC assay

zDEVD-AFC cleavage assay was performed using the manufacturer's protocol. Briefly, 5×10^6 cells were treated with galectin-1, buffer control, anti-Fas mAb or media for 3 h at 37°C. Cells were washed with PBS and extracted with 0.2 ml lysis buffer (1 mM PMSF, 5 mM DTT, 25 mM HEPES pH 7.5, 0.1% Triton-X100, 10% glycerol), and cell extracts were added to assay buffer (50 mM HEPES, pH 7.5, 10% sucrose, 0.1% CHAPS), 10 mM DTT, and 100 μM zDEVD-AFC, and incubated at 37°C for 1 h. For zDEVD-fmk controls, 50 μM zDEVD-fmk was added to this cocktail and preincubated for 1 h at 37°C before addition of zDEVD-AFC. Analysis was carried out on a Cytofluor plate reader (ex = 395, em = 530).

Measurement of mitochondrial membrane potential and mitochondrial cardiolipin

After incubation with the indicated agent, 2×10^5 cells were incubated with 40 nM $DiOC_6(3)$ to measure mitochondrial membrane potential, or 40 nM 10-*N*-nonyl acridine orange (NAO)[28] to measure mitochondrial cardiolipin, and PI for 15 min at room temperature prior to analysis by flow cytometry. Loss of mitochondrial membrane potential or of mitochondrial cardiolipin was indicated by decreased cellular staining with the respective dyes.

Confocal immunofluorescence microscopy

CEM cells were treated with galectin-1 or buffer control as above and aliquots were removed for flow cytometry analysis. Cells were washed in cold PBS, fixed in 1 ml 2% paraformaldehyde, PBS for 30 min on ice, and quenched with 3 ml 0.2 M glycine, PBS. Pelleted cells were permeabilized with 0.1% TritonX-100, washed with PBS, and blocked with 100 μl of 2% goat serum, 4 mg/ml BSA, PBS overnight at 4°C. Cells were incubated with rabbit anti-human EndoG antiserum (1:250) or rabbit anti-human AIF (1 : 100), and mouse anti-human cytochrome *c* mAb (1 : 100) in 2% goat serum, BSA, PBS, overnight at 4°C. After washing with 1% BSA/PBS, cells were incubated with FITC conjugated goat anti-rabbit mAb (1 : 100), and Texas Red conjugated goat anti-mouse mAb (1 : 100) for 1 h at 4°C. Alternatively, cells were stained with anti-EndoG or anti-AIF, and with PI (2.5 μg/ml) during addition of secondary antibody. Cells were washed with PBS, mounted on slides with Antifade mounting media and dried at RT overnight in the dark. Analysis was carried out on a Fluoview laser scanning confocal microscope, and images was processed with Fluoview imaging analysis software (Olympus America, Inc., Melville, NY, USA). To quantify labeled nuclei, at least 50 cells in five different fields were examined.

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