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# Perforin-dependent nuclear entry of granzyme B precedes apoptosis, and is not a consequence of nuclear membrane dysfunction

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## Abstract

**Killer lymphocytes utilize the synergy of a membranolytic protein, perforin, and the serine protease granzyme B (grB) to induce target cell apoptosis, however the mechanism of this synergy remains incompletely defined. We have previously shown that perforin specifically induces the redistribution of cytoplasmic grB into the nucleus of dying cells, however a causal role for nuclear targeting of grB in cell death has not been demonstrated. In the present study, we used confocal laser scanning microscopy (CLSM) to determine whether the nuclear accumulation of fluoresceinated (FITC-) grB precedes or is a consequence of apoptosis. Two distinct and mutually exclusive cellular responses were observed in FDC-P1 cells: (i) up to 50% of the cells rapidly accumulated FITC-grB in the nucleus (maximal at 7 min; $t_{1/2}$ of 2 min) and underwent apoptosis; (ii) the remaining cells took up FITC-grB only into the cytoplasm, and escaped apoptosis. Under these conditions, DNA fragmentation was not observed for at least 13 min, indicating nuclear accumulation of grB preceded the execution phase of apoptosis. Furthermore, nuclear import of grB proceeded through an intact nuclear membrane, as the nuclei of cells whose cytoplasm was pre-loaded with 70 kDa FITC-dextran excluded dextran for up to 90 min while still undergoing apoptosis in response to perforin and grB. These findings indicated that perforin-induced nuclear accumulation of grB precedes apoptosis, and is not a by-product of caspase-induced nuclear membrane degradation. The cell membrane lesions formed by perforin in these experiments were not large enough to permit a 13 kDa protein (yeast cdk $p13^{suc}$) access into the cytoplasm, but an 8 kDa protein (bacterial azurin) was able to equilibrate between the cytosol and the exterior. Therefore, transmembrane pores large enough to allow passive diffusion of grB (32 kDa) into the cell are not necessary for apoptosis. Rather, a perforin-dependent signal results in a redistribution of grB from the cytoplasm to the nucleus, where it may contribute to the nuclear changes associated with apoptosis.**

**Keywords:** granzyme; perforin; caspases; nuclear localization; cytolytic lymphocyte

**Abbreviations:** grB, granzyme B; ICE, interleukin-1$\beta$-converting enzyme; PARP, poly(ADP-ribose) polymerase; DNA-PKcs, catalytic subunit of DNA dependent protein kinase; CLSM, confocal laser scanning microscopy.

## Introduction

Killer cells such as CTL utilize two principal means of inducing an apoptotic signal in target cells. The first involves engagement of the receptor molecule, Fas/CD95 on the target cell by its ligand CD95L/FasL on the effector (Rouvier *et al*, 1993; Yonehara *et al*, 1989; Trauth *et al*, 1989). The second involves exocytosis of the contents of cytoplasmic granules from the killer cell toward the target (Young and Cohn, 1986; Smyth and Trapani, 1995). The apoptotic effects of cytolytic granules can be mimicked by exposing cells to just two molecules, the pore-forming protein perforin, and the serine protease granzyme B (grB) (Shi *et al*, 1992). Neither perforin nor grB is able to induce apoptosis in the absence of the other agent (Shi *et al*, 1992; Duke *et al*, 1989). Accordingly, perforin-deficient mice exhibit defective cytolysis against virus-infected, allogeneic and tumor targets (Kagi *et al*, 1994; Lowin *et al*, 1994), and the killer lymphocytes of mice lacking grB induce delayed cell death (Heusel *et al*, 1994).

There has been a great deal of recent interest in the mechanism of grB/perforin synergy, but even the precise sites of action of the two molecules are yet to be agreed. Perforin, a membranolytic agent, inserts in the target cell membrane (Ortaldo *et al*, 1992) and is not known to enter the cytoplasm. Cells whose cytoplasm is preloaded with certain protease inhibitors may withstand CTL attack (Nakajima and Henkart, 1994) through the inhibition of apoptotic cysteine proteases similar to the *Caenorhabditis elegans* protein, CED-3 (Sarin *et al*, 1997). GrB cleaves at specific aspartic acid residues (Poe *et al*, 1991), and its

substrates include interleukin-1$\beta$-converting enzyme (ICE) and multiple other apoptotic cysteine proteases. These include proteases both near the apex of the cascade (Mch5/Mch1a/FLICE and Mch4; Boldin *et al*, 1995, 1996; Fernandex-Alnemri *et al*, 1996), and CPP32 (Caspase 3) (Fernandes-Alnemri *et al*, 1996; Darmon *et al*, 1995; Quan *et al*, 1996), which is further downstream. Activated CPP32 plays a key role in DNA fragmentation (Darmon *et al*, 1996) by inactivating poly(ADP-ribose) polymerase (PARP[1]) (Lazebnik *et al*, 1994; Nicholson *et al*, 1995; Tewari *et al*, 1995; Fernandes-Alnemri *et al*, 1996) and the catalytic subunit of DNA-dependent protein kinase (DNA-PKcs) (Song *et al*, 1996; Kaufmann *et al*, 1993). Direct activation of Mch2a by grB also results in the cleavage of structural nucleoproteins such as lamins (Srinivasula *et al*, 1996). Recent evidence suggests grB may also directly cleave PARP (Froelich *et al*, 1996a) and DNA-PKcs (Song *et al*, 1996) at sites distinct from those of the CED-3-like proteases. Thus, both grB and the caspases may be capable of contributing to nuclear apoptotic morphology.

We (Trapani *et al*, 1996; Jans *et al*, 1996; Shi *et al*, 1997) and others (Pinkowski *et al*, 1996) have demonstrated the capacity of grB to localize within the cytoplasm of target cells independently of perforin, indicating that perforin-induced pore-formation does not adequately explain the cellular uptake of apoptotic proteases such as grB. Nevertheless, cells that have taken up grB remain indefinitely viable in the absence of perforin. The co-addition of perforin induces apoptosis, and this is accompanied by a pronounced redistribution of grB from the cytoplasm to the nucleus of the dying cell (Trapani *et al*, 1996; Shi *et al*, 1997). Perforin may therefore play a key role in 'redeploying' grB from some shelted compartment such as an edosome [as was postulated recently by Froelich *et al* (1996a)] to the cytosol, where it might now access its substrates in both the cytoplasm and nucleus. Despite the appeal of this hypothesis, a causal relationship between nuclear accumulation and apoptosis has not been established. It is possible that entry of grB into the nucleus is not a primary event, rather a mere consequence of prior nuclear membrane damage, perhaps as a result of the immediate activation of the caspase cascade. To address these issues, we performed a careful quantitative and kinetic analysis of grB's intracellular distribution during apoptosis, using confocal laser-scanning microscopy (CLSM). Our results clearly demonstrate that perforin-induced redistribution of grB to the nucleus *precedes*, and is invariably followed by nuclear apoptotic changes eventually culminating in cell death. These observations are consistent with the notion that in addition to activating key cytoplasmic pathways, grB may contribute directly to the distinctive nuclear changes of apoptotic cell death.

## Results

### Sublytic concentrations of perforin induce nuclear uptake of grB

FDC-P1 cells were exposed to perforin and FITC-grB and observed by CLSM (Figure 1A). Two populations of cells became evident within 2–4 min: in about 50% of the cells, the cytoplasm and nucleus became fluorescent, and this staining was predictive of ensuing apoptotic death. The remainder of the cells also took up grB, but in lower amounts, and appeared to be spared from apoptosis. In the stronger stained population, grB was localized principally within the nucleus as compared to the cytoplasm (Figure 1A right panel). At early time points ($<$20 min), neither population showed apoptotic features: the outline of the nucleus was smooth and regular, and fluorescence was distributed uniformly throughout all of the nuclei (Figure 1).

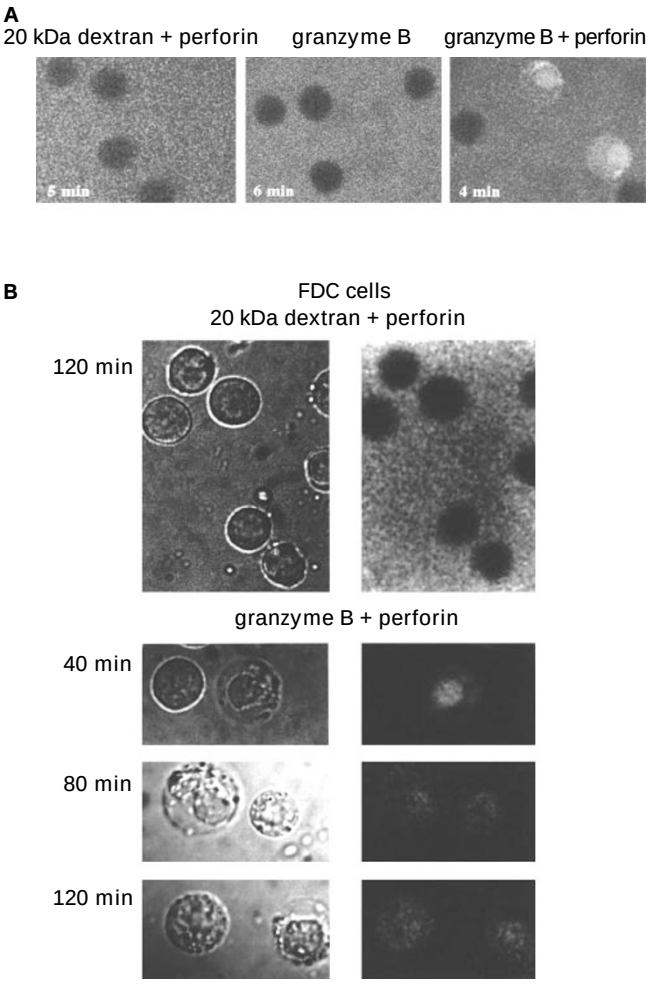


**Figure 1** (**A**) Uptake and nuclear accumulation of FITC-grB by FDC-P1 cells is dependent on perforin. Cells were exposed to either FITC-dextran (20 kDa) in the presence of perforin (left), or FITC-grB in the absence (centre) or presence (right) of perforin at 37°C, and fluorescence visualized at the times indicated using CLSM. The experiment depicted was typical of 12 similar experiments performed over a range of grB concentrations (0.5 to 10 $\mu$g/ml) with perforin at 50–200 U/ml. (**B**) Progressive changes in morphology and nuclear staining in response to perforin and FITC-grB. At 40 min, the typical apeparance of adjacent apoptotic and non-apoptotic cells exposed to perforin and grB is shown under phase contrast microscopy (left), with the corresponding fluorescent confocal image shown at right. At 80 and 120 min, other cells show progressive collapse and nuclear disintegration, with loss of nuclear definition. Control cells exposed to FITC-dextran and perforin are shown at 120 min. Note that most of the FITC-grB remains extracellulr and the cell shape uniform

We have previously observed nuclear and nucleolar accumulation of FITC-grB in semi-intact rat hepatoma cells (Trapani *et al*, 1996). However, in the present study, FITC-grB was not preferentially localized in the nucleolus. Cells exposed to the same quantity of FITC-grB in the absence of perforin were similar in appearance to the weakly-stained population exposed to both reagents. By 5–6 min, comparatively small amounts of FITC-grB were taken up into the cytoplasm, and extracellular fluorescence remained correspondingly higher than in cells additionally exposed to perforin (Figure 1A). Nuclear staining was not seen in cells exposed to FITC-grB alone, or in the population exposed to both agents that did not undergo apoptosis.

To exclude the possibility that entry of grB (32 kDa) might simply reflect cell membrane disruption due to perforin pore-formation, FDC-P1 cells were exposed to perforin in the presence of 20 kDa FITC-dextran (Figure 1A, left panel and Table 1). The FITC-dextran was not taken up by cells, suggesting that entry of grB under these conditions was not due to 'non-specific' perforin-induced membranolysis. Indeed, cells exposed to FITC-dextran and unlabeled grB together with perforin were just as susceptible to apoptosis, but excluded FITC-dextran for up to 60 min (Table 1, and data not shown). We have made similar observations for cellular exclusion of 20 kDa FITC-dextran in the presence of perforin from HTC rat hepatoma cells (Jans *et al*, 1996) and mouse YAC-1 lymphoma cells ($Fc/Fmed_{max}$=0.45, unpublished observations). We also wished to determine whether the membrane lesions induced by perforin under these conditions were sufficiently large to permit the entry of molecules smaller than 20 kDa. We therefore examined the cytoplasmic and nuclear uptake of two inert proteins, the 13 kDa subunit of a yeast cdk ($p13^{suc}$) and bacterial azurin (8 kDa) in the presence or absence of perforin and/or grB (Table 1). $p13^{suc}$ showed no cytoplasmic accumulation when applied with perforin (Fc/Fmed=0.28). An increase in this ratio was observed for azurin (from background levels, 0.36, up to 0.92), suggesting that the perforin lesions were just large enough to allow entry into the cell. Neither protein could inhibit apoptosis when applied with both perforin and unlabeled grB. Cell death under these conditions was associated with uptake of both $p13^{suc}$ and azurin into the cytoplasm in the cells undergoing apoptosis (Fc/Fmed 1.25 and 1.12, respectively), but not in those that survived. These results indicated that the perforin lesions formed under these conditions were conducive to apoptosis, induced the redistribution of grB from the cytoplasm to the nucleus, yet did not permit the free passage of molecules as small as 13 kDa into the cells. More precise definition of the size of the lesions effected by perforin will require examination of several other proteins in the 8–12 kDa range, using the sorts of approaches described here.

**Table 1** Cellular and nuclear uptake of various molecules in FDC-P1 cells in the presence or absence of perforin and/or grB

| Molecule | Addition (s)[1] | $(Fc/Fmed)_{max}$[2] | $(Fn/c)_{max}$[2] |
|---|---|---|---|
| FITC-grB | None | 1.20 | 0.83 |
| (32 kDa) | Perforin | 2.72 (1.40)[3] | 1.60 (0.56)[3] |
| FITC-dextran grB | | 0.76 | 0.73 |
| (20 kDa) | Perforin | 0.39 | 0.60 |
| | Perforin+grB | 0.98 (0.51)[3] | ND[4] |
| FLUOS-$p13^{suc}$ | None | 0.45 | 0.56 |
| (13 kDa) | Perforin | 0.28 | 0.73 |
| | Perforin+grB | 1.25 (0.52)[3] | ND[4] |
| FLUOS-azurin | None | 0.36 | 0.84 |
| (8 kDa) | Perforin | 0.92 | 0.66 |
| | Perforin+grB | 1.12 (0.65)[3] | ND[4] |

[1]Treatment with unlabeled protein. [2]The data for maximal cellular ($Fc/Fmed_{max}$) or nuclear ($Fn/c_{max}$) accumulation represent the mean of least two separate experiments (S.E.M. $<$ 12% of the mean). [3]Values in parentheses indicate those for 'non-apoptotic' cells (see text). [4]Not Determined

## Nuclear uptake of FITC-grB in cell populations undergoing apoptosis

FDC-P1 cells exposed to FITC-grB with or without perforin were observed under fluorescence microscopy, and simultaneously using CLSM in transmission mode to detect cells undergoing apoptosis (Figure 1B). After 25–40 min, only the cells with nuclear fluoresence (but none of the cells without nuclear fluorescence) began to show clear morphological evidence of apoptosis. These cells became highly refractile and the nuclei misshapen and irregular. Cell volume typically increased for a few minutes, and then contracted. When viewed under fluorescence, these nuclei initially became progressively brighter and condensed, but eventually disintegrated and their fluorescence became indistinguishable from cytoplasm (Figure 1B). By contrast, cells that excluded grB from the nucleus invariably remained alive. In the typical experiment shown (Figure 2A), very few apoptotic cells were seen in the first 25 min of exposure to perforin and FITC-grB. From that time onwards, the number of apoptotic cells continued to rise until the experiment ended at 4 h. Apoptotic cells were also estimated by TUNEL staining and by binding of FITC-conjugated annexin V (Koopman *et al*, 1994) (data not shown). Both techniques confirmed that death was induced only when both perforin and grB were present, and numbers of dead cells by these criteria closely paralleled morphological observations. In the experiment shown, FDC-P1 cells exposed to perforin and FITC-dextran (Figure 2A), or to perforin, FITC-grB or buffer alone (not shown) demonstrated fewer than 4% apoptotic cells throughout. CLSM in conjunction with image analysis was used to quantitate the subcellular uptake of FITC-grB. A time-course of nuclear accumulation of FITC-grB in the strongly stained and weakly stained cells exposed to perforin and FITC-grB is shown (Figure 2B). Only the cells undergoing apoptosis demonstrated significant nuclear accumulation of grB ($Fn/c > 1$). In the typical experiment shown, nuclear acumulation of grB was very rapid: Fn/c (fluorescence in the nucleus relative to that in the cytoplasm) reached maximal levels (1.60) at 7.5 min with a $t_{1/2}$ of $\sim$2 min. Fn/c did not exceed 0.5 in non-apoptotic cells. We have obtained very similar quantitative results using either HTC (Jans *et al*, 1996) or YAC-1 cells. In the latter case, the maximal Fn/c values for grB in the absence and presence of perforin were 0.9 and 2.0, respectively (unpublished observations).

## Kinetics of grB nuclear uptake

In cells exposed to both FITC-grB and perforin for 10 min, the brightly-staining population had taken up 2.5 to 3 times more grB into the cytoplasm than cells that did not undergo apoptosis (Figure 3). By 3 h, this ratio had increased progressively to ~5. Initially, cells exposed to FITC-grB alone were very similar in appearance and fluorescence intensity with the non-apoptotic cells exposed to both reagents. Over the next 3 h, however, cells exposed to grB alone progressively took up moderate quantities of FITC-grB, but did not undergo cell death. Accumulation in the nucleus only occurred in the population exposed to both perforin and grB that was undergoing (or about to undergo) apoptosis (Fn/c of about 1.5). Our results indicate clear differences in the cellular uptake and subcellular distribution of grB in apoptotic and non-apoptotic populations, both in the total amount taken up (Figure 3) and its subcellular distribution (Figure 2B).

## Nuclear entry of grB is not due to disruption of the nuclear envelope

To confirm that in the experiments described above, grB was not entering the nucleus secondary to nuclear membrane damage, we added perforin and unlabeled grB to FDC-P1 cells whose cytoplasm was pre-loaded with FITC-labeled 70 kDa dextran (Figure 4, left panel). The nuclei of these cells excluded the fluorescent label for up to 90 min (Fn/c ~0.5), irrespective of the presence of perforin and/or grB, implying that the nuclear envelope was intact even until late stages of apoptosis. Osmotic loading had not altered their sensitivity to grB in the presence of perforin, as mock-loaded cells exposed to FITC-grB and perforin rapidly accumulated grB in their nuclei, the Fn/c reaching maximal levels (Fn/c of 1.8–2.0) in less than 45 min (Figure 4, right panel). Thus, grB entry into the nucleus did not result from non-specific nuclear membrane damage incurred during apoptosis, and as has been shown previously in other systems (Earnshaw, 1995; Casciola-Rosen *et al*, 1995), the nuclear envelope remained intact even until late in apoptosis.

## Kinetics of apoptosis-induced DNA fragmentation

To more stringently ascertain if nuclear accumulation of grB preceded apoptosis, FDC-P1 cells were exposed to perforin and grB and the time course of DNA breakdown during apoptosis was examined by TUNEL (Figure 5). Since the incorporation of FITC-deoxyuridine into nicked DNA was measured, unlabeled grB was used in these studies. No DNA fragmentation above background levels (~2%) was seen

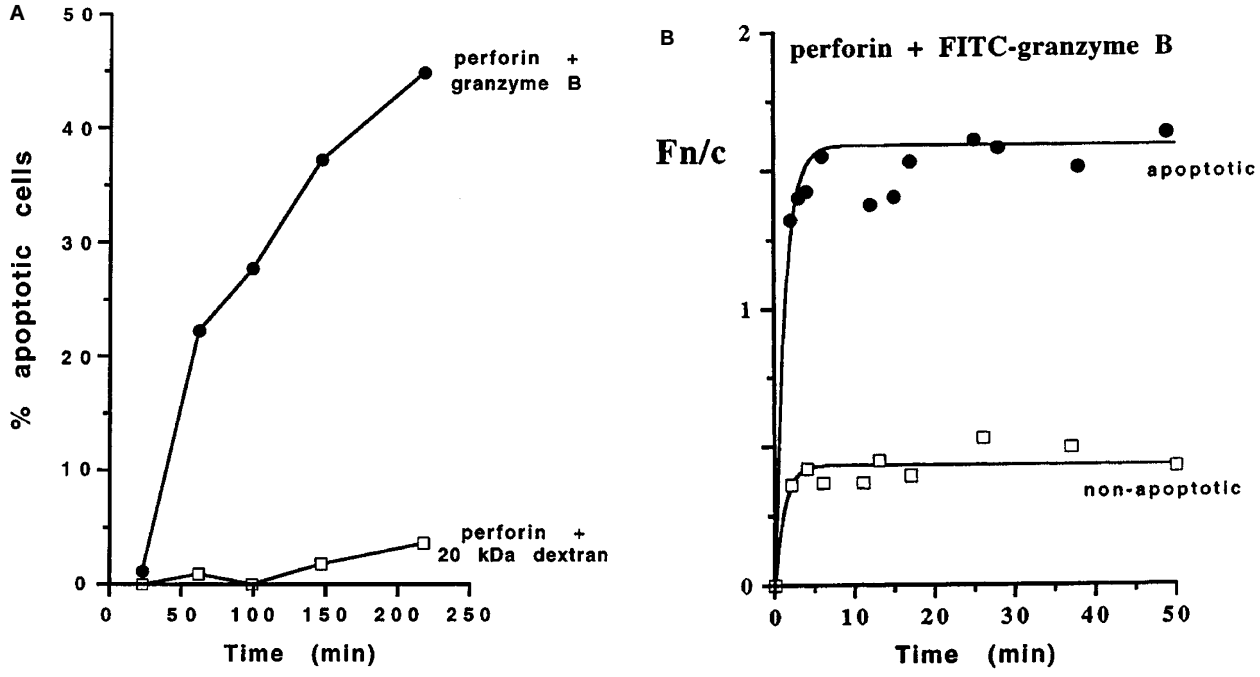


**Figure 2** (**A**) Time course of apoptosis induced by grB and perforin. FCD-P1 cells were exposed to 100 U/ml perforin together with either FITC-dextran (20 kDa), or FITC-grB at 37°C as indicated. Cells with typical apoptotic morphology were enumerated visually (~200 cells per time point). Background levels of apoptosis in cells exposed to perforin alone, FITC-grB alone or to buffer alone were <4% in this experiment (not shown). The experiment depicted was typcial of ten similar experiments performed over a range of grB concentrations (1–5 $\mu$g/ml). (**B**) Kinetics of nuclear accumulation of FITC-grB in apoptotic and non-apoptotic FDC-P1 cells. Cells were treated as in **A**, mounted and examined using CLSM in fluorescence and phase-contrast modes. Image analysis was performed to quantitate cellular (Fc/medium) and nuclear (Fn/c) uptake (see Materials and Methods). Results represent at least six separate meeasurements for each of the fluorescence measurements (Fn, Fc and autofluorescence), with the S.E.M. less than 4.2% the value of the mean. Exponential curve fitting was performed as described previously (27,28), with values for maximal nuclear uptake ($Fn/c_{max}$) being 1.59 and 0.43 for apoptotic and non-apoptotic cells respectively (regression coefficients of 0.91 and 0.94 for the curve fits, respectively). Maximal accumulation was achieved in the case of apoptotic cells within 7.5 min ($t_{1/2}$ ~2 min). Apoptotic and non-apoptotic cells were differentiated as described in the text

after 10 min exposure to perforin and grB, but by 20 min approximately 32% of the cells had fragmented DNA (Figure 5). In further experiments (not shown), we determined that DNA fragmentation was never observed prior to 13 min, i.e. at least 11 min after grB was first observed in the nucleus (Figure 1). As expected, DNA fragmentation was not

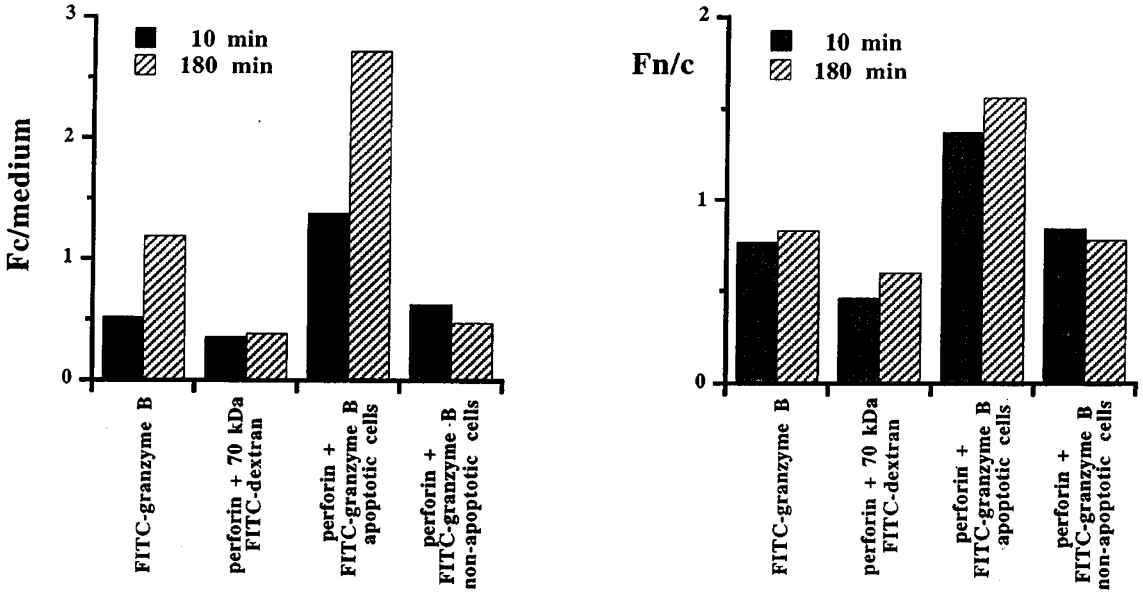


**Figure 3** Cellular uptake and nuclear accumulation of FITC-grB by apoptotic and non-apoptotic FDC-P1 cells in the absence and presence of perforin. Cells were treated as described in the legend to Figure 1. Image analysis was performed to quantitate cellular (Fc/medium) and nuclear (Fn/c) uptake. Results, shown for two time points for a single typical experiment, represent at least five separate measurements for each of the fluorescent measurements (Fc, Fmedium, Fn and autofluorescence), with the S.E.M. $<6.2\%$ of the mean. Apoptotic and non-apoptotic cells were differentiated on the basis of morphological criteria (see text)

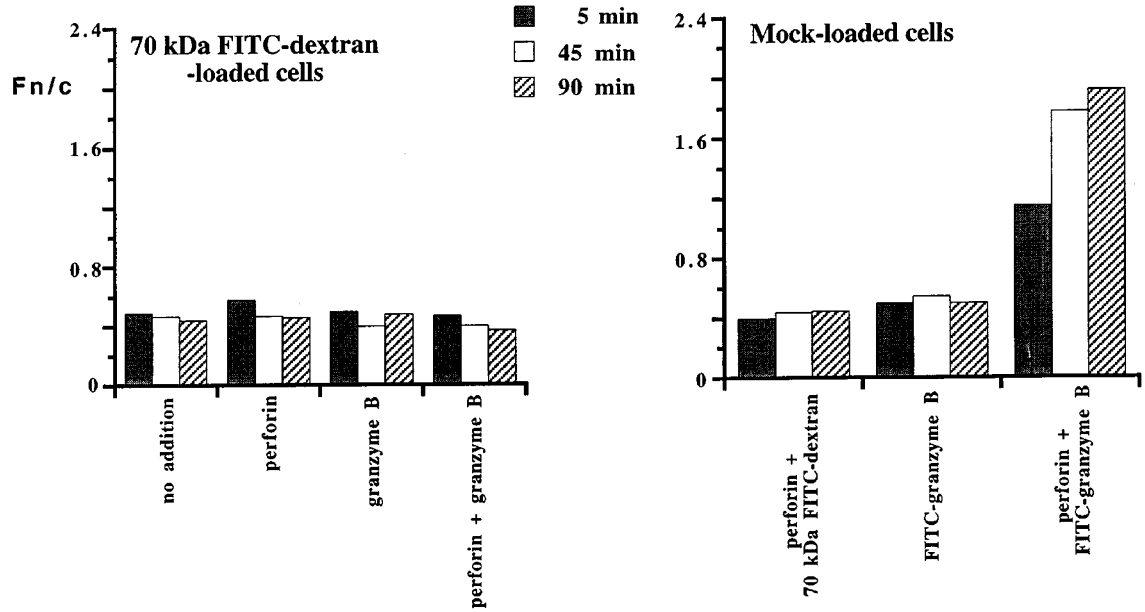


**Figure 4** Nuclear membrane integrity in FDC-P1 cells in the presence of grB/perforin. Cells were cytoplasmically loaded with FITC-dextran (70 kDa) or mock-loaded by hypertonic treatment as described (Nakajima and Henkart, 1994), prior to examination using CLSM and image analysis as described in the legend to Figure 3. Results, shown for three time points for a single typical experiment, represent at least four separate measurements for each of the fluorescent measurements (Fc, Fn and autofluorescence), with the S.E.M. $<9.9\%$ of the mean. Cells pre-loaded with a 20 kDa FITC-dextran in the same experiment yielded an Fn/c of 1.01 (equal concentration in cytoplasm and nucleus) at 5 min (not shown)

observed with either perforin or grB alone (Figure 5) or with 20 kDa dextran and perforin (data not shown). The onset of DNA fragmentation therefore closely paralleled the appearance of apoptotic cells as described above (Figures 1 and 2). Overall, our results strongly suggest that grB accumulation in the nucleus preceded apoptosis, as defined by DNA breakdown or by morphological criteria.

## Discussion

Several striking findings arose from the present study examining the intracellular distribution of grB in target cells exposed to perforin and grB as a model of CTL attack. We have clearly established by a variety of means that nuclear accumulation of grB preceded apoptosis, and was not simply a consequence of nuclear changes induced by apoptosis *per se*. In the presence of perforin, grB was very rapidly transported to the nucleus, with half-maximal levels achieved as rapidly as 2 min, the shortest time in which cells could be imaged, and at least 11 min before single-stranded breaks could be detected in DNA. Significantly, by comparing the simultaneous fluorescence and phase contrast appearances of many hundreds of cells, it was apparent that no cell that took up grB into its nucleus was spared from apoptosis. We have obtained very similar data using rat HTC or mouse Yac-1 cells (unpublished results), which indicates that our findings are not unique to FDC-P1 cells. The integrity of the nuclear membrane late in apoptosis was confirmed by our experiments in which the cytoplasm was loaded with 70 kDa FITC-dextran using the technique of Nakajima and Henkart (1994). Although some redistribution of nuclear pores occurs early in many forms of apoptosis (Earnshaw, 1995), the nuclear membrane usually remains impermeable to macromolecules until much later (Casciola-Rosen *et al*, 1995). Consistent with this, specifically degraded proteins, including PARP, remain intranuclear even after degeneration into discrete envelope-enclosed 'apoptotic bodies' (Casciola-Rosen *et al*, 1995). We have also shown previously that grB itself does not influence the passive permeability or active transport properties of the nuclear envelope (Trapani *et al*, 1996). Why only a subset of the FDC-P1 cells should be susceptible to perforin and grB is unclear. It now appears clear that cells are susceptible to the effects of grB and perforin throughout all stages of the cell cycle (Shi *et al*, 1996). We are currently examining possible parameters of resistance to apoptosis, including relative resistance to lysis by perforin, and differences in the uptake of grB.

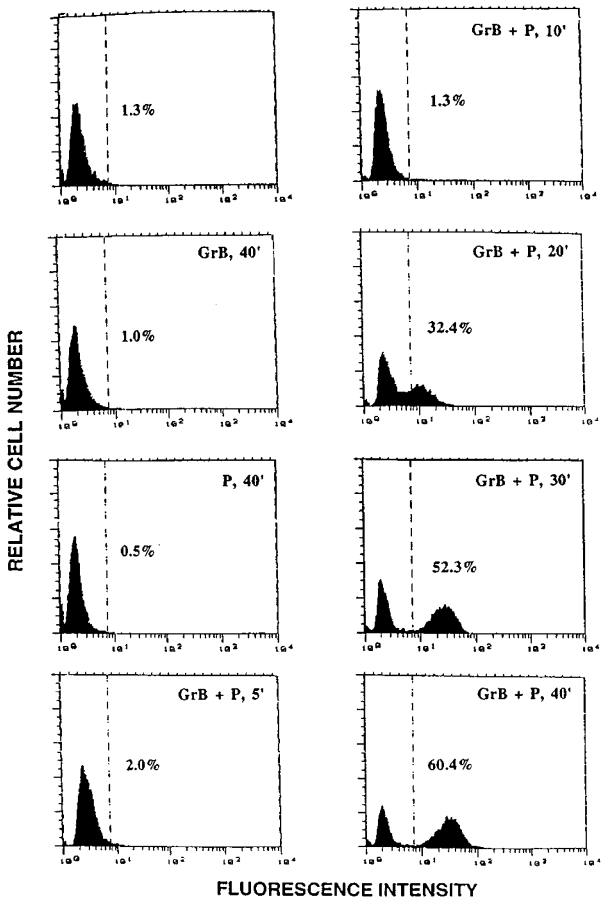


**Figure 5** Time course of DNA fragmentation in response to grB and perforin using TUNEL. FDC-P1 cells were incubated in the presence of buffer alone, or with perforin (P) and/or unlabeled grB for the times indicated at 37°C. DNA fragmentation was assayed using TUNEL staining and cytofluorography. The percentages of cells with DNA fragmentation are indicated. The results shown are for a single typical experiment, from a series of three similar experiments

It still remains to be determined whether granzymes (especially grB) contribute directly to intranuclear proteolysis in intact cells. We have unequivocally documented grB within the nucleus well before the classical nuclear changes of apoptosis take place, and this is consistent with grB contributing directly to nuclear damage. Nuclear proteins that can be cleaved by grB *in vitro* include PARP and DNA-PKcs, and the preferred cleavage sites (asp residues) for grB on these molecules are distinct from those utilized by caspases (Song *et al*, 1996; Froelich *et al*, 1996a). In addition, grB has been shown to bind an undefined nucleoprotein of 85 kDa (Pinkoski *et al*, 1996), while granzyme A, which can also be targeted to the nucleus of intact cells in the presence of perforin (unpublished observations) is capable of binding and cleaving the nuclear shuttle protein nucleolin (Pasternack *et al*, 1991). Therefore, granzymes may be directed to the nucleus to contribute to the proteolysis induced by caspases or their downstream effector molecules such as DFF (Liu *et al*, 1997).

We have shown here using FITC-tagged grB, and elsewhere using immuno-electron microscopy (Shi *et al*, 1997) that grB can be taken up into cells in the absence of perforin, pointing to the inadequacy of the hypothesis that polyperforin pores allow the passive diffusion of grB into the cell. However, the appearance of large amounts of grB in the cytoplasm and nuclei of cells undergoing apoptosis may be a consequence of, and a witness to the release of 'free' grB into the cytosol by perforin. It is likely that grB is taken up by intact cells in a vesicular form, and perforin may enable it to access the cytoplasm, perhaps by disrupting endocytic vesicles in a manner akin to adenovirus (Froelich *et al*, 1996b). This would allow grB to both activate the caspase cascade in the cytoplasm

(thereby causing indirect nuclear damage by generating a battery of nucleolytic proteases), and to additionally target its own nuclear substrates directly. Furthermore, both we and others (Sarin *et al*, 1997) have recently shown that synthetic caspase inhibitors such as Z-VAD-fmk can abolish both the nuclear uptake of grB and nuclear apoptotic changes, indicating that an intact caspase cascade in mandatory for the nucleolytic effect of grB (unpublished observations).

Our findings clearly indicate that perforin can facilitate nuclear trafficking of grB in the case of CTL-mediated cell death; the intriguing possibility that it may do the same for endogenous proteases such as activated caspases is currently under investigation. There is abundant evidence that many structural and enzymatic nuclear proteins are cleaved during apoptosis (Kumar and Lavin, 1996), and the proteases that target these molecules are not constitutively in the nucleus, so at least some must be transported to their sites of action, presumably through nuclear pore complexes. Although the primary structure of perforin has been known for many years (Shinkai *et al*, 1988; Lichtenheld *et al*, 1988), the nature of the membrane signal delivered by perforin also remains elusive: the uptake of grB into the cell is clearly not explicable in terms of the formation of non-selective transmembrane pores, and there is no clear evidence for activation of generic membrane signaling pathways by perforin. The mechanism by which grB traverses the cell membrane in either the absence or presence of perforin is also unknown. In the present study, the addition of perforin increased the overall uptake of grB into the cell (2–3-fold that in its absence). Our observations are consistent with those of Shi *et al* (1992) who demonstrated that DNA fragmentation could proceed in the presence of minute (sublytic) quantities of perforin, provided the perforin signal was accompanied by grB. There is no evidence for a specific perforin receptor other than lipid molecules with phosphorylcholine headgroups (Tschopp *et al*, 1989), nor is is clear how perforin might influence the overall uptake of grB into the cell. Although we can reconstitute nuclear import of grB in semi-intact cells using cytosolic extracts (Trapani *et al*, 1996; Jans *et al*, 1996), we have been unable to reconstitute the apoptotic signal of perforin with other membrane disruptive agents including complement, calcium ionophores, phorbol esters or mild detergent treatment (Shi *et al*, 1997 and unpublished data). The key to unraveling the precise function/s of perforin clearly awaits the development of systems to study structure/function relationshisp for the various domains of this intriguing molecule.

## Materials and Methods

### Cells

Mouse FCD-P1 myeloid cells were cultured in DMEM supplemented with 10% fetal calf serum and recombinant IL-3-containing culture supernatant (Karasuyama and Melchers, 1988).

### Protein purification and labeling

Immunoaffinity purified human grB was free of other granzymes and perforin as demonstrated previously (Trapani *et al*, 1993). GrB was labeled with FITC (Molecular Probes) as described (Trapani *et al*, 1996). This resulted in a $<20\%$ reduction in cleavage of the synthetic tripeptide substrate Boc-ala-ala-asp-S-benzyl (Trapani *et al*, 1996). FITC grB was equally active as unlabeled grB, in $^{51}Cr$ release assays in the presence of perforin. Protein concentrations of grB were determined using the theoretical extinction coefficient of 1.20 (Gill and von Hippel, 1989). Microtiter assays for grB activity were as described (Trapani *et al*, 1994). Rat perforin was purified as described (Froelich *et al*, 1996b). The cdk subunit $p13^{suc}$ was expressed in *E. coli* strain BL21(DE3)pLysS and purified essentially as described (Labbe *et al*, 1991). $p13^{suc}$ was labeled using FLUOS (Boehringer Mannheim), 150 $\mu$g of which was dissolved in 450 $\mu$l of DMSO and mixed with 2 mg of $p13^{suc}$ dissolved in 1 ml of 0.1 M sodium bicarbonate, pH 8.5. After 90 min at room temperature, unbound dye was removed from the labeled protein by chromatography on Sephadex G25.

### Cellular uptake and distribution of fluoresceinated molecules

FDC-P1 cells (Vaux *et al*, 1992) were harvested in the logarithmic phase of growth, washed three times and resuspended ($4 \times 10^6$/ml) in Hank's buffered saline solution containing 10 mM Hepes pH 7.2, 2 mM $CaCl_2$, 0.4% BSA and 0.1% (v/v) IL-3-containing culture supernatant (Buffer A) (Shi *et al*, 1992). Cells (6 $\mu$l) were incubated for the specified times with an equal volume of perforin (100–1000 U/ml, final concentration) and/or FITC-grB (0.25–10 $\mu$g/ml, final concentration), or with an equivalent amount of unlabeled grB. Perforin and grB were diluted and mixed immediately before the assay in 10 mM Hepes, 150 mM NaCl, 1 mM EGTA, pH 7.2. The reaction mixture was immediately pipetted onto a glass slide as described (Trapani *et al*, 1996). The dose of perforin produced $<5\%$ specific release of $^{51}Cr$ from FDC-P1 cells. In some experiments, FITC-labeled 20 kDa dextran (Sigma), $p13^{suc}$ or azurin were added with perforin. FITC-dextran addition had no inhibitory effect on perforin-induced $^{51}Cr$ release from FDC-P1 (data not shown).

Cells were scored as non-apoptotic or apoptotic based on microscopic examination (see Results section). The validity of this approach was confirmed by TUNEL analysis (compare Figures 2A and 5), and propidium iodide staining and electron microscopy (data not shown). Visualization and quantitation of the cellular and nuclear uptake of FITC-grB and the other FITC-labeled compounds was carried out using CLSM as described previously (Jans *et al*, 1991; Walaschewski *et al*, 1995; Xiao *et al*, 1996), and in particular for FITC-labeled grB (Trapani *et al*, 1996; Jans *et al*, 1996). Image analysis and curve fitting was carried out as described (Trapani *et al*, 1996; Ymer and Jans, 1995). Results were expressed in terms of Fc/medium (cellular uptake parameter; fluorescence quantitated in the cytoplasm–Fc–relative to fluorescence quantitated in the medium–Fmedium–following subtraction of background fluorescence), and Fn/c (nuclear accumulation; fluorescence quantitated in the nucleus–Fn–relative to Fc, following subtraction of background fluorescence) (Trapani *et al*, 1996; Ymer and Jans, 1995). In some experiments, CLSM was performed using a transmission detector in order to provide phase contrast and fluorescent images of the same fields.

### Cytoplasmic loading of FDC-P1 cells with FITC-dextran

Cells were washed twice and resupended in 0.1 ml of hypertonic solution (0.5 M sucrose, 10% polyethylene glycol 1000, 20 mM Hepes,

pH 7.2) (Nakajima and Henkart, 1994) containing 10 mg/ml FITC-labeled 20 or 70 kDa dextran at 37°C for 10 min. The medium was then made hypotonic by adding 7.9 ml of 60% RPMI medium diluted in water. Following a further 10 min, the cells were washed twice in Buffer A and resuspended at $4 \times 10^6$/ml. Mock-loaded cells were incubated in hypertonic buffer lacking FITC-dextran.

### TUNEL assay

Cells undergoing DNA fragmentation were enumerated with a *TUNEL* (terminal deoxyribonucleotidyl transferase labeling of DNA strand breaks with dUTP) kit purchased from Boehringer-Mannheim (Sydney, Australia), used according to the manufacturer's instructions. Cells were analyzed immediately on a cytofluorograph (FACScan, Becton-Dickinson).

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## References

Boldin MP, Goncharov TM, Golstev YV and Wallach D (1996) Involvement of MACH, a novel MORT1/FADD-interacting protease, in Fas/APO-1- and TNF receptor-mediated cell death. Cell 85: 803 – 815

Boldin MP, Varfolomeev EE, Pancer Z, Mett IL, Camonis JH and Wallach D (1995) A novel protein that interacts with the death domain of fas/apo1 contains a sequence motif related to the death domain. J. Biol. Chem. 270: 7795 – 7798

Casciola-Rosen LA, Anhalt GJ and Rosen A (1995) Specific cleavage of the 70 kdal component of the U1 small nuclear ribonucleoprotein is a characteristic biochemical feature of apoptotic cell death. J. Exp. Med. 182: 1625 – 1634

Darmon AJ, Nicholson DW and Bleackley RC (1995) Activation of the apoptotic protease CPP32 by cytotoxic T-cell-derived granzyme B. Nature 377: 446 – 448

Darmon AJ, Ley TJ, Nicholson DW and Bleackley RC (1996) Cleavage of CPP32 by grazyme B represents a critical role for granzyme B in the induction of target cell DNA fragmentation. J. Biol. Chem. 271: 21709 – 21712

Duke RC, Persechini PM, Chang S, Liu C-C, Cohen JJ and Young JD-E (1989) Purified perforin induces target cell lysis but not DNA fragmentation. J. Exp. Med. 170: 1451 – 1456

Earnshaw WC (1995) Nuclear changes in apoptosis. Curr. Opin. Cell Biol. 7: 337 – 343

Fernandes-Alnemri T, Armstrong R, Krebs J, Srinivasula SM, Wang L, Bullrich F, Fritz L, Tomaselli KJ, Trapani JA, Croce C, Litwack G and Alnemri ES (1996) Activation of CPP32 and Mch3 by Mch4, a novel human apoptotic cysteine protease containing two FADD-like domains. Proc. Natl. Acad. Sci. USA 93: 7464 – 7469

Froelich CJ, Hanna WL, Poirier GG, Duriez PJ, D'Amours D, Salvesan GS, Alnemri ES, Earnshaw WC and Shah GM (1996a) Granzyme B/perforin mediated apoptosis of Jurkat cell results in cleavage of ploy(ADPribose) polymerase to the 89 kDa apoptotic fragment and a less abundant 64 kDa fragment. Biochem. Biophy. Res. Commun. 227: 658 – 667

Froelich CJ, Orth K, Turbov J, Seth P, Gottlieb R, Babior B, Shah GM, Bleackley RC, Dixit VM and Hanna W (1996b) New paradigm for lymphocyte granule mediated cytotoxicity: target cells bind and internalize granzyme B but an endosomolytic agent is necessary for cytosolic delivery and subsequent apoptosis. J. Biol. Chem. 271: 29073 – 29079

Gill SC and von Hippel PM (1989) Calculation of protein extinction coefficients from amino acid sequence data. Analytical Biochem. 182: 319 – 326

Heusel JW, Wesselschmidt RL, Shresta S, Russell JH and Ley TJ (1994) Cytotoxic lymphocytes rquire granzyme B for the rapid induction of DNA fragmentation and apoptosis in allogeneic target cells. Cell 76: 977 – 987

Jans DA, Ackermann M, Bischoff JR, Beach DH and Peters R (1991) $p34^{cdc2}$-mediated phosphorylation at $T^{124}$ inhibits nuclear import of SV40 T-antigen proteins. J. Cell Biol. 115: 1203 – 1212

Jans DA, Jans P, Briggs LJ, Sutton V and Trapani JA (1996) Reconstitution of nuclear and nucleolar transport of the natural killer cell serine protease grazyme B-dependence on perforin in vivo and cytosolic factors in vitro. J. Biol. Chem. 271: 30781 – 30789

Kagi D, Ledermann B, Burki K, Seiler P, Odermatt B, Olsen KJ, Podack ER, Zinkernagel RM and Hengartner H (1994) Cytotoxicity mediated by T cells and natural killer cells is greatly impaired in perforin-deficient mice. Nature 369: 31 – 35

Kaufmann SH, Desnoyers S, Ottaviano M, Davidson NE and Poirier GG (1993) Specific proteolytic cleavage of poly (ADP-ribose) polymerase: an early marker of chemotherapy-induced apoptosis. Cancer Res., 53: 3976 – 3985

Karasuyama H and Melchers F (1988) Establishment of mouse cell lines which constitutvely secrete large quantities of interleukin 2, 3, 4 or 5, using modified cDNA expression vectors. Eur. J. Immunol. 18: 97 – 102

Koopman G, Reutelinsperger CP, Kuitjen GA, Keehnan RM, Pals ST and van Oers MH (1994) Annexin V for flow cytometric detection of phosphatidylserine expression on B cells undergoing apoptosis. Blood 84: 1415 – 1420

Kumar S and Lavin MF (1996) The ICE family of cysteine proteases as effectors of cell death. Cell Death Differ. 3: 255 – 267

Labbe J-C, Cavadore J-C and Doree M (1991) M-phase specific cdc2 kinase – preparation from starfish oocytes and properties. Methods Enzymol. 200: 291 – 301

Lazebnik YA, Kaufmann SH, Desnoyers S, Poirier GG and Earnshaw WC (1994) Cleavage of poly (ADP-ribose) polymerase by a proteinase with properties like ICE. Nature 371: 346 – 347

Lichtenheld MG, Olsen KJ, Lu P et al (1988) Structure and function of human perforin. Nature 335: 448 – 451

Lowin B, Beermann F, Schmidt A and Tschopp J (1994) A null mutation in the perforin gene impairs cytolytic T lymphocyte- and natural killer cell-mediated cytotoxicity. Proc. Natl. Acad. Sci. USA 91: 11571 – 11575

Liu X, Zou H, Slaughter C and Wang X (1997) DFF, a heterodimeric protein that functions downstream of caspase-3 to trigger DNA fragmentation during apoptosis. Cell 89: 175 – 184

Nakajima H and Henkart PA (1994) Cytotoxic lymphocytes trigger a target cell disintegration pathway leading to cytolysis and DNA breakdown. J. Immunol. 152: 1057 – 1063

Nicholson DW, Ali A, Thornberry NA, Villaincourt JP, Ding CK, Gallant M, Garaeau Y, Griffin PR, Labelle M, Lazebnik YA, Munday NA, Raju SM, Smulson ME, Yamin T-T, Yu VL and Miller DK (1995) Identification and inhibition of the ICE-CED-3 protease necessary for mammalian apoptosis. Nature 376: 37 – 43

Ortaldo JR, Winkler-Pickett RT, Nagashima K, Yagita H and Okumura K (1992) Direct evidence for release of pore-forming protein during NK cellular lysis. J. Leukoc. Biol. 52: 483 – 488

Pasternack MS, Bleier KJ and McInerney TN (1991) Granzyme A binding to target cell proteins. Granzyme A binds to and cleaves nucleolin in vitro. J. Biol. Chem. 266: 14703 – 14708

Pinkoski MJ, Winkler U, Hudig D and Bleackley RC (1996) Binding of granzyme B in the nucleus of target cells. Recognition of the 80 kilodalton protein. J. Biol. Chem. 271: 10225 – 10229

Poe M, Blake JT, Boulton DA, Gammon N, Sigal NH, Wu JK and Zweernik HJ (1991) Human cytotoxic lymphocyte granzyme B: its purification from granules and the characterization of substrate and inhibitor specificity. J. Biol. Chem. 266: 98 – 103

Quan LT, Tewari M, O'Rourke K, Dixit V, Snipas SJ, Poirier GG, Ray C, Pickup DJ and Salvesan GS (1996). Proteolytic activation of the cell death protease Yama/CPP32 by granzyme B. Proc. Natl. Acad. Sci. USA 93: 1972 – 1976

Rouvier E, Luciani MF and Golstein P (1993) Fas involvement in $Ca^{2+}$-independent T cell-mediated cytotoxicity. J. Exp. Med. 177: 195 – 200

Sarin A, Williams MS, Alexander-Miller MA, Berzofsky JA, Zacharchuk CM and Henkart PA (1997) Target cell lysis by CTL granule exocytosis is independent of ICE/Ced-3 family proteases. Immunity 6: 209 – 215

Shi L, Chen G, He D, Bosc DG, Litchfield DW and Greenberg AH (1996) Granzyme B induces apoptosis and cyclin A-associated cyclin-dependent kinase (cdk) activity in all stages of the cell cycle. J. Immunol. 157: 2381 – 2385

Shi L, Kraut RP, Aebersold R and Greenberg AG (1992) A natural killer cell granule protein that induces DNA fragmentation and apoptosis. J. Exp. Med. 175: 553 – 565

Shi L, Mai S, Israels S, Browne K, Trapani JA and Greenberg AH (1997) Granzyme B (GraB) autonomously crosses the cell membrane and perforin initiates apoptosis and GraB nuclear localization. J. Exp. Med. 185: 855 – 866

Shinkai Y, Takio K and Okumura K (1988) Homology of perforin to the ninth component of complement (C9). Nature 334: 525 – 527.

Smyth MJ and Trapani JA (1995) Granzymes: exogenous proteinases that induce target cell apoptosis. Immunol. Today 19: 202 – 206

Song Q, Burrows S, Lees-Miller S, Smith G, Jackson S, Kumar S, Trapani JA, Alnemri E, Litwack G, Lu H, Moss D and Lavin M (1996) ICE-like protease cleaves DNA-dependent protein kinase in cytotoxic T-cell killing. J. Exp. Med. 184: 619 – 628

Srinivasula S, Fernandes-Alnemri T, Zangrilli J, Robertson N, Armstrong RC, Wang L, Trapani JA, Tomaselli KJ, Litwack G and Alnemri ES. (1996) J. Biol. Chem. 271: 27099 – 27106

Tewari M, Quan LT, O'Rourke K, Desnoyers S, Zens Z, Beidler DR, Poirier GG, Salvesan GS and Dixit V (1995) Yama/CPP32b, a mammalian homologue of CED-3, is a crmA-inhibitable protease that cleaves the death substrate poly(ADP-ribose) polymerase. Cell 81: 801 – 809

Trapani JA, Browne KA, Smyth MJ and Jans DA (1996) Localization of granzyme B in the nucleus: a putative role in the mechanism of cytotoxic lymphocyte-mediated apoptosis. J. Biol. Chem. 271: 4127 – 4133

Trapani JA, Browne KA, Dawson M and Smyth MJ (1993) Purification of functional asp-ase (natural killer granzyme B) using a monoclonal antibody. Biochem. Biophys. Research Commun. 158: 910 – 920

Trapani JA, Smyth MJ, Apostolidis VA, Dawson M and Browne KA (1994) Granule serine proteases are normal nuclear constituents of natural killer cells. J. Biol. Chem. 269: 18359 – 18365

Trauth BC, Klas C, Peters AM, Matzku S, Molelr P, Falk W, Debatin KM and Krammer PH (1989) Monoclonal antibody-mediated tumor regression by induction of apoptosis. Science 245, 301 – 305

Tschopp J, Schafer S, Masson D, Peitsch MC and Heusser C (1989) Phosphorylcholine acts as a $Ca^{2+}$-dependent receptor molecule for lymphocyte perforin. Nature 337: 272 – 274

Vaux DL, Aguila HL and Weissman IL (1992) Bcl-2 prevents death of factor deprived cells but fails to prevent apoptosis in targets of cell mediated killing. Int. Immunol. 4: 821 – 827

Walaschewski U, Jans DA, Neugebauer-Goedde D-C and Peters R (1995) Semi-intact cells for nucleo-cytoplasmic transport studies. Methods in Mol. Cell. Biol. 5: 87 – 95

Xiao C-Y, Hübner S, Elliot RM, Caon M and Jans DA (1996) A consensus PK-A site in place of the CcN motif casein kinase II site of SV40 large T-antigen confers PK-A-mediated regulation of nuclear import. J. Biol. Chem. 271: 6451 – 6457

Ymer S and Jans DA (1995) In vitro Chromatin structure of the murine interleukin-5 gene region: a new intact cell system (1996) Biotechniques 20: 834 – 840

Yonehara S, Ishii A and Yonehara K (1989) A cell killing monoclonal antibody (ANTI-Fas) to a cell surface antigen co-downregulated with the receptor for tumor necrosis factor. J. Exp. Med. 169: 1747 – 1756

Young JD-E and Cohn ZA (1986) Cell-mediated killing: a common mechanism? Cell 46: 641 – 648