

# Granzyme A, a Stealth Killer in the Mitochondrion

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**The induction of caspase-independent cell death by killer lymphocytes involves the serine protease granzyme A (GzmA). In this issue, Martinvalet et al. (2008) show that GzmA penetrates the mitochondrial matrix without perturbing normal mitochondrial functions. In the mitochondrial matrix, GzmA cleaves NDUF3 (a component of the electron transport chain) leading to production of reactive oxygen species and ultimately to cell death.**

Defeating pathogens and eliminating transformed cells is the purpose of the immune system. One mechanism by which immune cells fulfill this function is to exploit the evolutionarily conserved capacity of cells to undertake a suicide program. Several mechanisms of programmed cell death (PCD) or apoptosis exist and often need to be triggered simultaneously for infected cells or tumor cells to be eliminated. Indeed, cellular transformation is generally associated with mutations in components of PCD pathways, and viruses have evolved mechanisms to interfere with PCD programs to prolong survival of their host cells. The onset of PCD in infected or transformed cells can be triggered by the release of serine proteases called granzymes by killer lymphocytes (Cullen and Martin, 2008). In this issue of *Cell*, Martinvalet and colleagues explore the mechanism linking granzyme A (GzmA) to the production of reactive oxygen species (ROS). ROS formation is a hallmark of apoptosis but is generally a late event following permeabilization of the mitochondrial outer membrane. Recent work showed that ROS generation can result from the translocation of active caspase-3 into mitochondria and the cleavage of NADH dehydrogenase (ubiquinone) Fe-S protein 1 (NDUFS1), another component of complex I of the respiratory chain (Ricci et al., 2004). Martinvalet et al. (2008) now show that GzmA rapidly penetrates the mitochondrial matrix and cleaves NADH dehydrogenase (ubiquinone) Fe-S protein 3 (NDUFS3), disrupting the electron transport chain and leading to ROS production and cell death.

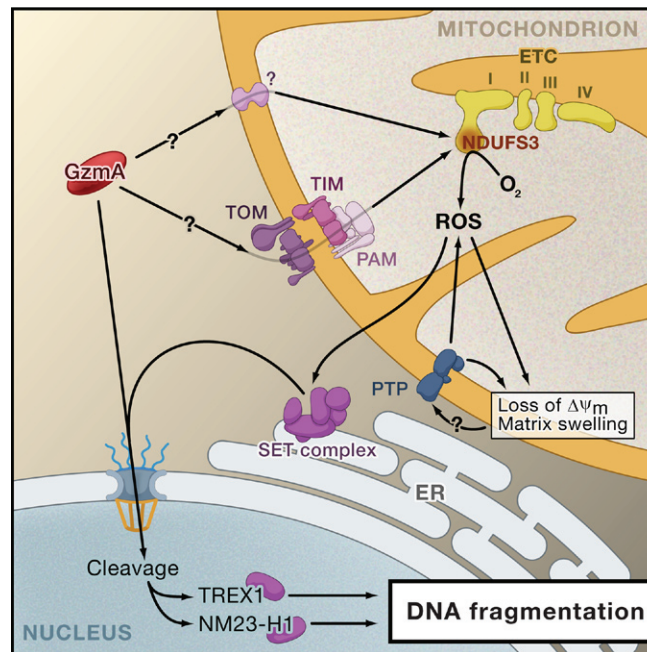
Natural killer (NK) cells and cytotoxic T lymphocytes (CTLs) are specialized cells of the immune system that recognize foreign antigens presented by antigen-presenting cells in association with class I major histocompatibility complex (MHC) proteins. The onset of PCD programs in infected or transformed cells is triggered through the activation of plasma membrane death receptors (in CTLs) or the release of granzymes (in both CTLs and NK cells). Granzymes are secreted at the immunological synapse (formed between NK cells and target cells) in perforin-containing granules and penetrate the cytosol of target cells by a mechanism still under debate. GzmB kills cells through a caspase-dependent pathway mediated either by the cleavage of the Bcl-2 protein Bid or by the direct proteolysis of caspases. In contrast, GzmA-mediated killing does not depend on caspases and is not inhibited by prosurvival members of the Bcl-2 family. A previous study by Martinvalet and colleagues indicated that GzmA may increase ROS production and that GzmA-mediated cell killing can be prevented by superoxide scavengers (Martinvalet et al., 2005). The production of ROS was found to promote the translocation of the ER-associated complex (SET complex) into the nucleus where its cleavage by GzmA induces the release of NM23-H1 and TREX1, two proteins responsible for the DNA fragmentation that contributes to GzmA-induced cell death (Figure 1). Although the downstream consequences of ROS production are known, the mechanism by which GzmA increases ROS production remains unclear.

In their new study, Martinvalet et al. (2008) used proteomic analysis of isolated mouse liver mitochondria after incubation with recombinant GzmA to identify putative mitochondrial targets of GzmA action. Among these, only NDUFS3, a core component of complex I of the respiratory chain, was cleaved upon incubation of human cells with a combination of perforin and GzmA. Moreover, in vitro assays with recombinant proteins indicated that NDUFS3 is a direct substrate of GzmA. This was an unexpected finding given that GzmA lacks any predicted mitochondrial targeting sequence and would have to penetrate deep into the mitochondria to reach its target.

NDUFS3 is a poorly characterized component of complex I of the mitochondrial respiratory chain. NDUFS3 mutations are associated with late-onset Leigh syndrome, a neurological disease linked to mutations in other core components of complex I (Benit et al., 2004). Despite this connection, the role of NDUFS3 in the mitochondrial respiratory chain is unclear. NDUFS3 has no predicted transmembrane domain and is therefore likely to be localized in the matrix portion of this multisubunit complex (Hirst et al., 2003). In agreement with their in vitro data showing the direct cleavage of NDUFS3 by GzmA, Martinvalet and colleagues now demonstrate using immunoelectron microscopy and import assays on isolated mitochondria that GzmA is in fact able to enter the mitochondrial matrix. Furthermore, they show that the import of GzmA occurs via a pathway dependent on the mitochondrial transmembrane potential ( $\Delta\Psi_m$ ). Indeed,

disruption of the  $\Delta\Psi_m$  inhibited NDUFS3 cleavage and interfered with ROS production. The authors also showed an association of GzmA with the chaperones Hsp70 and Hsp90, which have been implicated in the mitochondrial import. Although Hsp90 did not copurify with mitochondrial components, some cytosolic Hsp70 was associated with the mitochondrial surface and thus could contribute to the partial unfolding of GzmA required for its import through the Tom and Tim mitochondrial protein translocation complexes (Kutik et al., 2007) (Figure 1). These data suggest that GzmA specifically translocates into the mitochondrial matrix by a mechanism that does not perturb the normal function of mitochondria. This is in contrast to the passive diffusion of caspase-3 into the mitochondrial intermembrane space during apoptosis, after outer mitochondrial membrane permeabilization (Ricci et al., 2004). The identity of the signals and proteins that trigger and mediate GzmA import remains unknown. An increasing number of proteins lacking mitochondrial targeting signals are being found in mitochondria. These proteins may be either targeted to the Tom complexes of the outer mitochondrial membrane by alternative unidentified targeting signals or imported via some yet undiscovered translocation machinery.

In their previous paper, Martinvalet et al. (2005) also reported the inhibition of GzmA-induced ROS production and cell death by inhibitors of the permeability transition pore (PTP). Opening of the PTP, a putative multisubunit complex spanning both mitochondrial membranes, potentiates ROS production through the dissipation of the mitochondrial proton gradient. Moreover, it induces matrix swelling and could therefore be responsible for the abnormal mitochondrial morphology that is a characteristic of GzmA-induced cell death (Martinvalet et al., 2008). PTP opening could thus be a late event trig-



**Figure 1. Granzyme A-Induced ROS Production and Cell Death**

After entering the cytosol of target cells, granzyme A (GzmA) is specifically imported into mitochondria either through the Tim/Tom/Pam import pathway for matrix proteins or through an unknown mechanism. In the mitochondrial matrix, GzmA cleaves NADH dehydrogenase (ubiquinone) Fe-S protein 3 (NDUFS3), a component of complex I of the electron transport chain (ETC), inducing production of reactive oxygen species (ROS). ROS formation could be further amplified by opening of the permeability transition pore (PTP), which could also be responsible for GzmA-induced swelling of the mitochondrial matrix and loss of mitochondrial transmembrane potential ( $\Delta\Psi_m$ ). Increased ROS in the cytoplasm of cells promotes translocation of the ER-associated complex (SET complex) into the nucleus, where its cleavage by GzmA induces the release of NM23-H1 and TREX1, two proteins responsible for DNA fragmentation that contribute to GzmA-induced cell death.

gered by the loss of transmembrane potential and of ATP production. It may also result from the association of GzmA with mitochondrial chaperones, which were recently suggested to control PTP opening (Kang et al., 2007).

In their new work, Martinvalet et al. (2008) show that expression of a mutant NDUFS3 protein resistant to GzmA-mediated cleavage specifically inhibited GzmA-induced ROS production and cell death. However, it has recently been reported that NDUFS3 is upregulated in cancer cells when they are exposed to cell death-inducing compounds and that depletion of NDUFS3 by a short-interfering RNA prevented apoptosis and reduced ROS production (Huang et al., 2007). But how can both NDUFS3 cleavage and increased NDUFS3 expression (Huang et al., 2007) be observed to associate with ROS production? It will be interesting to determine whether

the expression of the cleaved protein is sufficient to induce ROS production and cell death, or whether cleavage of additional mitochondrial proteins by GzmA is necessary.

Intriguingly, GzmB has also been shown to induce caspase-independent mitochondrial ROS production (Martinvalet et al., 2005). GzmB does not cleave NDUFS3 but may function by targeting complex I of the respiratory chain, a mechanism that would also require its import into the mitochondrial matrix. As complex I is found at the beginning of the electron transport chain and is a site of ROS formation, it is ideally positioned to regulate both energy production and cell death. In fact, altering the expression of complex I subunits and their localization may be mechanisms used by viruses for proliferation, either through the promotion of ROS formation that induces infected cell death and virions release (Ladha et al., 2005) or to sustain the cellular production of energy required for viral replication (Reeves et al., 2007). Identifying the mecha-

nisms used by granzymes to perturb mitochondrial function therefore might shed light on strategies used by pathogens to escape host defenses and to survive and proliferate.

Diffusion of proteins out of the mitochondria is known to be a central step in the apoptotic program in response to stress. However, killing by a reverse mechanism, that is, by the entry of a protein into mitochondria to disrupt the electron transport chain to provoke increased ROS production, is an exciting discovery. The study by Martinvalet and coworkers now identifies the core components responsible for GzmA-induced cell death. Understanding precisely how GzmA reaches the mitochondrial matrix and how NDUFS3 cleavage is linked to ROS formation is the next step toward the comprehension of a key pathway involved in combating pathogens and eliminating cancer cells.

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# DeTEKting Ubiquitination of APC/C Substrates

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**Regulated protein degradation by the ubiquitin-proteasome pathway ensures the unidirectionality of mitotic progression by removing cell-cycle regulators required at earlier stages. The APC/C ubiquitin-protein ligase targets proteins by appending polyubiquitin degradation signals that are subsequently recognized by the 26S proteasome. Reporting in this issue, Jin et al. (2008) identify a TEK motif in both ubiquitin and substrates of APC/C that mediates assembly of these degradation signals.**

Cell-cycle progression relies on precise transitions through an ordered sequence of events coordinated in space and time. The intricate molecular dance of genome replication, spindle assembly, sister chromatid segregation, and cytokinesis is directed by the regulated action of cyclin-dependent protein kinases (Cdks). In eukaryotes, the forward progression of the cell cycle is propelled by the regulated degradation of cyclins and other proteins required at earlier steps in the cell cycle. Ubiquitin carrier proteins (E2-conjugating enzymes or Ubcs) act in concert with the anaphase-promoting complex/cyclosome (APC/C) and the Cdc20 targeting adaptor protein to assemble polyubiquitin degradation signals on cyclins, thus signaling destruction of these regulatory oscillators by the 26S proteasome. The APC/C similarly engages the Cdh1 targeting adaptor protein to initiate anaphase by licensing the degradation of securin, an inhibitor of the

ubiquitin-independent protease known as separase that initiates sister chromatid segregation by degrading their cohesin protein linker. Thus, regulated protein degradation by the ubiquitin/26S proteasome pathway serves as a common thread that links the coordinated action of the cyclins with other cell-cycle regulators (reviewed in Peters, 2006). Work by Jin et al. (2008) reported in this issue now reveals the mechanism by which APC/C directs assembly of polyubiquitin degradation signals.

Polyubiquitin chains can be formed by the conjugation of ubiquitin molecules to one another via any of the seven lysine residues that ubiquitin contains. However, because only a subset of these polyubiquitin chains can target proteins to the proteasome, productive ubiquitination by the APC/C must exhibit specificity in the type of ubiquitin-chain linkage formed upon polyubiquitination. Previous work has demonstrated

by quantitative mass spectrometry that the APC/C<sup>Cdc20</sup> complex of the frog *Xenopus* enlists the E2 Ubch10 to assemble degradation-competent polyubiquitin chains in vitro on cyclin B1 (Kirkpatrick et al., 2006). These polyubiquitin chains not only contain the canonical degradation-signaling chain linkage of lysine 48 (K48) (25%) but also chain linkages of lysine 11 (K11) (60%) and lysine 63 (K63) (15%). To further examine chain selectivity, Jin et al. (2008) evaluated APC/C ubiquitination in human cell extracts harboring mutant ubiquitin in which only a single lysine or all but one lysine were changed to arginine, a residue to which ubiquitin cannot be conjugated. Using cyclin B1 or securin ubiquitination and degradation as the functional readout of ubiquitin chain formation, the authors demonstrated that human APC/C<sup>Cdc20</sup> and APC/C<sup>Cdh1</sup> form predominantly (but not exclusively) K11-linked chains. Surprisingly, these chains are competent