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Divergence towards a dead end? Cleavage of the divergent domains of ribosomal RNA in apoptosis

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Abstract. In several cases of apoptotic death the large ribosomal subunit 28S rRNA is specifically cleaved. The cleavages appear at specific sites within those domains of the rRNA molecule that have shown exceptional high divergence in evolution (D domains). The cleavages accompany rather than precede apoptosis, and there is a positive, but not complete, correlation between rRNA cleavage and internucleosomal DNA fragmentation. Most cell types studied so far show two alternative cleavage pathways that are mutually exclusive. Cleavage can either start in the D8 domain with secondary cuts within a subdomain of D2 (D2c), or in the D2 domain with subsequent excision of the D2c subdomain. The latter pathway is of particular interest since D2 (unlike D8) is normally inaccessible for RNase attack. That apoptosis specifically affects the ribosomal divergent domains suggests that these domains, which make up roughly 25% of total cellular RNA, might have evolved to serve functions related to apoptosis. Future studies will be directed to test the hypothesis that rRNA fragmentation may be part of an apoptotic program directed against the elimination of illegitimate (viral?) polynucleotides.
Key words. Ribosome; divergent domains; apoptosis; rRNA cleavage; D2; D8.

Introduction

Macromolecular cleavage is central to apoptosis. Limited proteolysis may be pivotal in some apoptotic death-induction and death-execution pathways [1, 2, 3], and cleavage of chromosomal DNA leading to DNA fragmentation [4] was the first major molecular change to be discovered in conjunction with apoptosis [5]. Another form of apoptosis-associated polynucleotide cleavage, ribosomal RNA 'fragmentation', has been described more recently [6, 7, 8]. The apoptosis-associated rRNA cleavages occur in ribosomal divergent domains (D domains). These domains are made up of variable stretches of rRNA (ranging from 3 to 873 nucleotides in humans) that are interspersed between the conserved rRNA domains necessary for protein synthesis [9]. Although D domains make up roughly 25% of cellular RNA, their function is unknown, and apart from data on sequence and molecular evolution little is known about them. The aim of this article is briefly to summarize knowledge about the ribosomal divergent domains and their fate during apoptosis, in the hope that this will catalyse interest in these so far elusive ribosomal regions.

Selective cleavage of 28S ribosomal RNA in apoptosis

Ribosomal RNA cleavage during apoptosis selectively affects the large 28S rRNA molecule in the 60S ribosomal subunit, the 18S rRNA molecule in the 40S ribosomal subunit remaining unaffected [7]. Furthermore, unlike the apoptotic DNA cleavage that ultimately leads to the formation of small nucleosome-sized 0.3-kb fragments that leak out of the nucleus [10], the cleaved rRNA remains as an integral part of the ribosome (with the notable exception of the D2c domain, see below). When present, rRNA cleavage and internucleosomal DNA fragmentation follow immediately after the typical apoptoid changes in cellular morphology in all systems studied by us [8]. Thus, both DNA and rRNA cleavage appear to be part of the apoptotic death-execution pathway rather than of the apoptosis-induction pathway. In support of this view, microinjection of ribosomes with cleaved rRNA did not induce apoptosis in the recipient cell (Olav K. Vintermyr, unpublished observations), indicating that ribosomes from apoptotic cells are unable to transmit an apoptotic process to normal cells.

Fragmentation of rRNA and of DNA are most often linked [11, 8], but we have recently observed DNA fragmentation without rRNA cleavage in one cell line. Furthermore, in closely related cell lines (Molt-3 and Molt-4 leukemia cells) large subunit (LSU)-rRNA cleavage was found in one cell line and not in the other (A. Somali, O. K. Vintermyr, T. Cotter, S. O. Døskeland and G. Houge, unpublished observations). We hypothesize that coordinated cleavage of nuclear DNA and cytoplasmic rRNA is the rule in certain forms of apoptotic cell death, but that some cells may have lost components required to trigger or execute cleavage of either one or both types of polynucleotide. It is of interest that a dual function nuclease (DN/

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RNase) with both cytoplasmic and nuclear localization has been identified and shown to be proteolytically activated during apoptosis [12].

28S rRNA cleavage is not necessarily deleterious for protein synthesis

While extensive DNA fragmentation certainly must be deleterious for gene transcription, apoptotic LSU-rRNA cleavage does not necessarily disturb ribosomal function in protein synthesis. Even though total protein synthesis is dramatically decreased in apoptotic cells [13, 7, 14], this might have little to do with the occurrence of rRNA cleavage but could be a consequence of e.g. eIF2α-kinase activation or guanosine diphosphate (GDP) accumulation. We have been able to reconstitute the protein translational activity of lysates from highly apoptotic rat myeloid IPC-81 cells to levels approaching the level found in lysates made from normal cells by restoration of the adenosine triphosphate (ATP) level (T. S. Eikhom and G. Houge, unpublished observations), indicating that apoptotic rRNA cleavage leaves the protein synthetic ability of the ribosome at least partly intact. Furthermore, the occurrence of LSU-rRNA cleavage in polysomal as well as monosomal RNA also lends support to the idea that this phenomenon does not directly affect protein synthesis [8]. In addition, one should be aware that in the mitochondria of some unicellular organisms ribosomal RNA is naturally fragmented in six to eight pieces due to split or even scrambled ribosomal genes [15, 16, 17]. Despite the lack of covalent attachment to each other, the mitochondrial rRNA pieces can be modelled to form the conserved secondary structure typical for LSU-rRNA. The location of the known natural breaks in these mitochondrial LSU-rRNAs can vary from species to species, but all occur within the ribosomal divergent domains (see below).

The rRNA cleavage affects major 28S rRNA divergent domains

Ribosomal RNA consists of conserved and divergent (or variable) domains. The primary and secondary structure of the conserved domains are similar in all the three major kingdoms (eubacteria, archaebacteria and eukaryotes), and these conserved domains can be regarded as the 'core' RNA backbone of the ribosome, serving essential functions in protein synthesis. In archaebacteria the LSU-rRNA is slightly expanded compared to eubacterial LSU-rRNA, but large-scale expansion is only found in eukaryotic LSU-rRNA. These expansions of the rRNA molecule have taken place in regions that are inserted between the conserved domains, and because the primary structure (sequence) of the expansions is highly variable from species to species, they are called variable or divergent domains. Unfortunately, there is also variability in the numbering and naming of these domains [9]. Here we have chosen to use the nomenclature of Michot et al. [18] where the divergent domains are numbered from D1 to D12. The D domains have evolved within strict frames for secondary structure, indicating that only a certain secondary structure is compatible with D-domain function and/or that a structure that does not interfere with conserved ribosomal domains must be maintained [19, 20, 21]. In fact, there appears to be a strong negative selection against random mutations in the D domains, at least in *Drosophila* where the divergent domains are evolving at a slow rate relative to the rest of the rDNA and the genome as a whole [21]. This is a strong indication that the D domains have more than a structural role to play in the ribosome, possibly functioning as riboregulators, protein anchoring regions or domains for RNA/RNA interactions.

The two largest divergent domains, D2 and D8, are the ones usually cleaved in apoptosis [8]. Both domains have two apoptotic cleavage sites. An exception to this 'rule' is TNF/cycloheximide-induced apoptosis in bovine endothelial cells [8]. In this case cleavage occurs in D6 and D8. Whether this is due to some peculiarity of bovine D-domain secondary structure must await the sequencing of bovine LSU-rRNA.

The pattern of rRNA cleavage occurring during apoptosis cannot be reproduced in necrotic cells or when exposing cell lysates to random RNase activity [8]. It was found, however, that the D8 region was extensively cleaved as a prominent first step in these nonphysiological cases of ribosomal RNA degradation, but the cleavage sites appeared to be different (at least initially) from the D8 cleavage sites attacked in apoptosis. In contrast, neither D2 nor the D6 domain is a prime target for such RNase activity.

As mentioned above, the mitochondrial LSU-rRNA of some protista can be naturally fragmented. In the algae *Chlamydomonas reinhardtii* discontinuities are found in the regions corresponding to D2 and D6, but not in D8 [15]. The location of the break found in D2 roughly corresponds to the location of a break found in D2 of the LSU-rRNA of the dinoflagellate *Prorocentrum micans* [22]. In this case the break occurs in cellular (and not mitochondrial) rRNA, and it is not due to transcription of separate ('modularized') LSU ribosomal genes. This *P. micans* cleavage site occurs in the same stem-loop junction as the 3′D2 cleavage site found in apoptosis [8].

Two alternative 28S rRNA cleavage pathways coexist in apoptosis

The divergent domains so far implicated in apoptosis are more variable than other divergent domains [23, 24]

and the b-arm of D8 even has a short stretch of hypervariability, i.e. a stretch of nucleotides that can show great variation even in rRNA isolated from the same cell line [25]. There is an intriguing co-evolution of D2 and D8 with respect to both size and GC (guanine cytosine) content, suggesting a functional link between these domains [24]. A functional link is also suggested by the two alternative cleavage pathways that can be found in apoptosis [8]. The primary target for cleavage can either be D2 or D8. If cleavage starts in D8, secondary cleavage within a subdomain of D2 (D2c) occurs. In contrast, if cleavage starts in D2, a cleavage just 5′ to the D2c subdomain is followed by a cleavage just 3′ to D2c, resulting in excision of D2c from the ribosome. There is an apparent positive correlation between the proportion of primary D2 cleavage and the rapidity of onset of apoptotic cell death, at least in rat myeloid IPC-81 cells (G. Houge, unpublished observations). A schematic (and highly hypothetical) model for the interaction between D2 and D8 and the two alternative cleavage pathways is given in figure 1. In bovine endothelial cells alternative primary targets for rRNA cleavage are D6 or D8 [8].

Cleavage during apoptosis can either start in an RNase-sensitive domain (D8) or in a more RNase-protected region (D2 or D6). We expect the cleavage occurring in D8 to be due to activation of an (unknown) RNase or deprotection of this rRNA region by e.g. proteolysis or phosphorylation of associated protein(s).

The primary cleavages in D2 flanking D2c are most likely caused by ribosomal conformational changes leading to intrinsic ribozymatic activity or to exposure of the cleavage site to RNase, to give two examples. There are three reasons for speculating that D2c has an intrinsic capacity for self-excision. First, the cleavage sites in D2 occur in single-stranded regions that appear to be caged within the ribosome [26, 27]. Second, the cleavage sites are in stem-loop junctions [8], i.e. they have some structural resemblance to hammerhead ribozymes [28]. Third, a natural (spontaneous?) cleavage site can be found in the same location as the 3′D2c cleavage site in the dinoflagellate *Prorocentrum micans* [22].

Future perspectives

The phenomenon of rRNA fragmentation can give new information about the apoptotic process as well as about rRNA function. The ribosomal divergent domains, although constituting ~25% of cellular RNA, are still 'searching for a function'. It is highly unlikely that they are functionless. This is not only because it would be a waste of energy to make so much RNA for no purpose, but mainly because of their great conservation in secondary structure despite considerable variation at the sequence level [21, 24].

One potentially fruitful strategy for learning more about both apoptosis and rRNA is to search for specific apoptosis-associated RNases by comparing the rRNA cleavage pattern in cell lysates exposed to RNases with the pattern observed in intact cell apoptosis. Studies

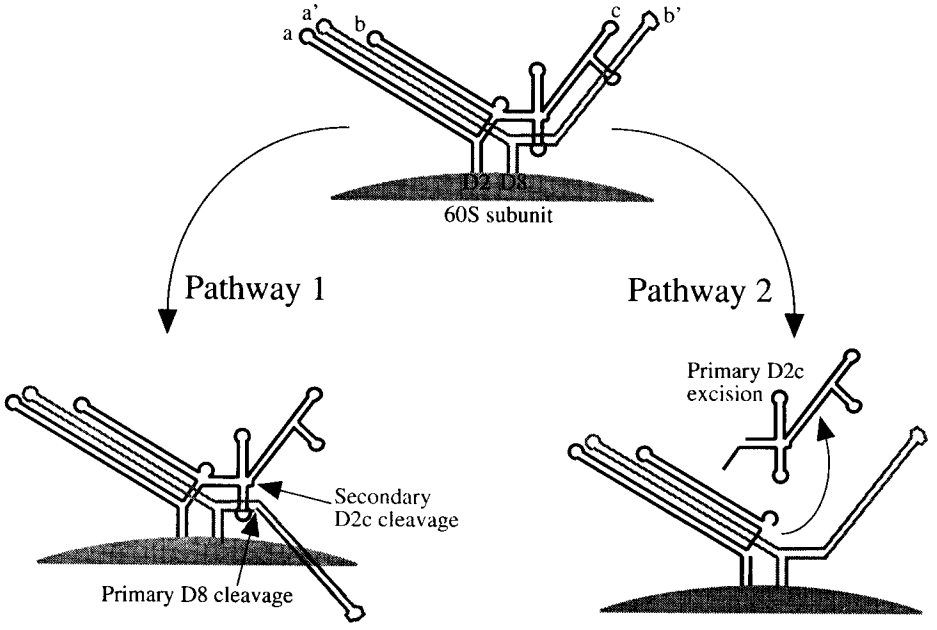

Figure 1. Two alternative pathways for apoptotic 28S rRNA cleavage. A schematic figure proposing a direct interaction between the ribosomal divergent domains D2 and D8. The three subdomains of D2 (black line) are named a, b and c and the two subdomains ('arms') of D8 (grey line) are called a′ and b′ [24]. The secondary structure depicted in the figure has been verified by electron microscopy (for a review, see Wakeman et al. [27]). The proposed interaction could explain why D2 and D8 co-evolve in size and GC content and why cleavage in D8 causes secondary cleavage within the c domain of D2 (pathway 1), a cleavage that is incompatible with primary D2c excision (pathway 2).

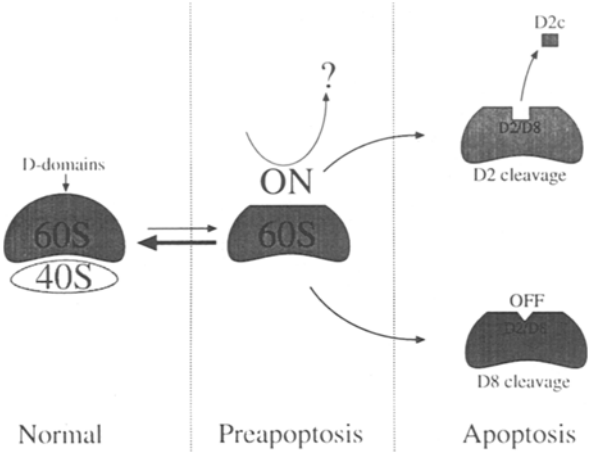

Figure 2. Hypothetical model for D-domain function in apoptosis. The abundance of ribosomes makes them ideal candidates for serving as cytoplasmatic 'biosensors' regulating cellular processes. It is speculated that various proapoptotic signal transduction pathways, e.g. involving phosphorylation and/or proteolysis, can convert D domains from a passive to an active (on) state. If a sufficient number of ribosomes are in the on-state, the threshold for apoptosis is exceeded. Secondary to these postulated D-domain modulations, apoptotic rRNA cleavage occurs which either inactivates the D domains or liberates D2c, which might have additional biological effects.

along these lines have just started in our laboratory. So far, no RNase has been found to reproduce in cell lysates the apoptotic cleavage pattern observed in intact cell apoptosis. Another strategy is to look for proteins protecting the specific rRNA cleavage sites against degradation. If putative protecting proteins are removed through reversible modification (like phosphorylation) or proteolysis in preapoptotic cells, it should be possible to achieve more extensive chemical modification of the thus deprotected rRNA. Using the technique of primer-extension analysis on chemically modified rRNA [26, 27], we have so far been unable to find structural alterations in the D8 domain of ribosomes isolated from preapoptotic cells. One should be cautious, however, in interpreting data derived from experiments where ribosomes have been removed from their natural cellular context. It still remains possible that a ribosome which is in a potential 'on-state' for apoptosis can quickly reverse to an 'off-state', as hypothetically modelled in figure 2. This type of experiment should therefore preferably be repeated using agents that can penetrate intact cells.

Another potentially fruitful strategy is to compare the functional differences between apoptotic processes in cells with marked rRNA fragmentation and cells without rRNA fragmentation. It is of special interest to know whether viral multiplication is halted in cells with extensive rRNA fragmentation. One potential antiviral mechanism could be that the process that leads to rRNA fragmentation also fragments viral RNA. Another mechanism could be that the excised D2c rRNA fragment or minor hypervariable D8 fragments liberated during apoptosis can have antiviral actions. Hopefully, future research will be able to shed more light on the significance of rRNA fragmentation in apoptosis and of the divergent domains of rRNA, which constitute one of the remaining black boxes of biology.

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