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A new face on apoptosis: death-associated protein 3 and PDCD9 are mitochondrial ribosomal proteins

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Abstract Two proteins known to be involved in promoting apoptosis in mammalian cells have been identified as components of the mammalian mitochondrial ribosome. Proteolytic digestion of whole mitochondrial ribosomal subunits followed by analysis of the peptides present using liquid chromatography–tandem mass spectrometry revealed that the proapoptotic proteins, death-associated protein 3 (DAP3) and the programmed cell death protein 9, are both components of the mitochondrial ribosome. DAP3 has motifs characteristic of guanine nucleotide binding proteins and is probably the protein that accounts for the nucleotide binding activity of mammalian mitochondrial ribosomes. The observations reported here implicate mitochondrial protein synthesis as a major component in cellular apoptotic signaling pathways.

Key words: Mitochondrion; Apoptosis; Ribosome; Ribosomal protein; Human

1. Introduction

Apoptosis or programmed cell death plays crucial roles in numerous biological processes ranging from growth and development to combating viral infections. Mitochondria play a key role in many pathways leading to cell death [1,2]. The precise mechanisms underlying the role of mitochondria in apoptosis remain to be elucidated [3]. However, it is clear that one important event is the release of cytochrome *c* and other proapoptotic proteins from the intermembrane space following initiation of a death pathway.

The mechanisms of a number of proteins shown to be involved in apoptosis remain unknown [4]. One proapoptotic protein of unknown function is the death-associated protein 3 (DAP3) [5–8]. DAP3 mediates interferon-, tumor necrosis factor- and FAS-induced cell death. When over-expressed, it causes apoptosis in a number of different types of mammalian cells [7,8]. Inactivation of the expression of DAP3 using antisense RNA protects HeLa cells from interferon-induced cell death, while over-expression of DAP3 induces apoptosis. Its precise role in the induction of cell death is not known although current data indicates that it functions downstream of the death-inducing signaling complex but upstream of the some members of the caspase family [7,8]. A second proapoptotic protein of unknown function is programmed cell death protein 9 (PDCD9 or p52). Expression of *Gallus gallus* (chicken) p52 in mouse fibroblasts causes apoptosis. In the current work, we demonstrate that both DAP3 and PDCD9 are previously uncharacterized mitochondrial ribosomal proteins. This observation suggests that components of the mitochondrial protein biosynthetic system may play a pivotal role in apoptosis.

2. Materials and methods

2.1. Preparation of bovine mitochondrial ribosomal proteins for 2D gel electrophoresis

Bovine mitochondria were prepared as described previously by Matthews et al. [9]. For analysis of individual proteins on 2D gels, samples were prepared as described [10,11]. For analysis of proteins in whole 28S subunit preparations, 55S mitochondrial ribosomes were isolated on sucrose gradients. The purified 55S ribosomes were dissociated into 28S and 39S subunits and the 28S subunits were purified on a second sucrose gradient [9].

2.2. Peptide sequencing by mass spectrometry (MS)

Spots from the 2D PAGE of the mitochondrial 28S subunits were digested in-gel with Endoprotease Lys-C (Boehringer Mannheim, Indianapolis, IN, USA) [12–14]. Nanoscale capillary liquid chromatography–tandem mass spectrometric (LC/MS/MS) analyses of in-gel digests were done using an Ultimate capillary LC system (LC Packings, San Francisco, CA, USA) coupled to a quadrupole time-of-flight mass spectrometer (Micromass, Manchester, UK) fitted with a Z-spray ion source as described previously [11].

For analysis of proteins in whole small subunits, 3 pmol of 28S subunits were subjected to endoprotease Lys C digestion [15]. The peptides produced were analyzed by variable flow LC/MS/MS (Blackburn et al., in preparation). Uninterpreted peptide product ion spectra and de novo sequenced peptides generated by LC/MS/MS were searched against the non-redundant protein database and human and EST databases for exact matches using the Mascot search program [16].

2.3. Computational analysis

Peptide sequences obtained from Mascot searches and those obtained by de novo sequencing from peptide product ion spectra were searched against the non-redundant protein database using the FASTA algorithm [17]. EST database and genomic DNA searches of the peptide sequences were performed using BLAST [18]. Sequence analysis and homology comparisons were done using the GCG software package (Wisconsin Package Version 10, 1999, Genetics Computer Group, Madison, WI, USA), Vector NTI (Informax Inc.) and Biology WorkBench 3.2. The results were displayed using BOXSHADE (version 3.21, written by K. Hofmann and M. Baron). Pre-

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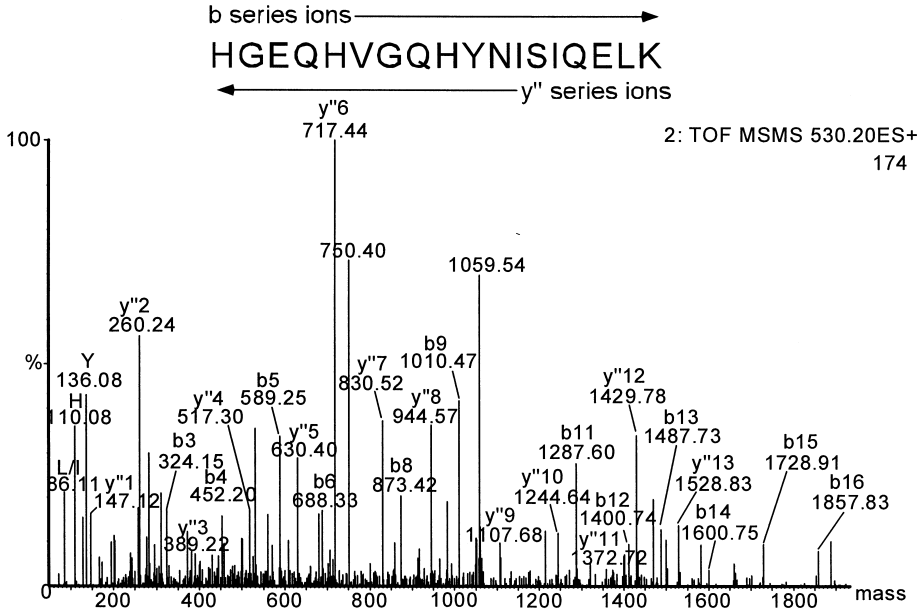

Fig. 1. Product ion spectrum of the Lys-C produced peptide at *m/z* 530.20 derived from DAP3. Proteins from the 28S subunit were digested with Lys-C and the peptides analyzed by LC/MS/MS [11].

diction of the cleavage sites for the mitochondrial signal sequence was carried out using PSORT and MitoProt II [19,20].

2.4. Analysis for prenylation in mitochondrial ribosomes

Gas chromatography (GC)/MS analyses were performed on a HP 5890 GC interfaced to a VG70-250SEQ mass spectrometer as described previously [21]. Selected ion monitoring (SIM) experiments at high resolving powers (RP 10 000) were performed by monitoring the ions, *m/z* 206.2034 (M^+•) and 191.1800 ($M–CH_3$)$^+$ for farnesene and the ions, *m/z* 274.2661 (M^+•) and 259.2426 ($M–CH_3$)$^+$ for geranyl geranene. The corresponding PFK lock-masses were 192.9888 and 268.9824, respectively. Standards for the GC/MS were synthesized from farnesyl bromide and all-*trans* geranyl geraniol by conversion to the bromide followed by treatment with superhydride [22,23]. The structures of chemically synthesized isoprenoid standards, farnesene (all-*trans*-2,6,10-trimethyl-2,6,10 dodecatriene) and geranyl geranene (all-*trans*-2,6,10,14-tetramethyl-2,6,10,14-hexadecatetraene) were confirmed by high resolution GC/MS.

3. Results and discussion

3.1. DAP3

Two experimental approaches provided information that DAP3 is a mitochondrial ribosomal protein. In the first approach, proteins extracted from bovine mitochondrial 28S subunits were separated on 2D gels [11]. The protein in one spot with a molecular mass of about 43 kDa and an isoelectric point of approximately 8.5–9 designated MRP-S29 was digested in-gel with endoprotease Lys-C and subjected to analysis by LC/MS/MS. In the second approach a complete mixture of proteins from the small subunit was digested with endoprotease Lys-C and the peptides obtained were analyzed by LC/MS/MS. A representative spectrum obtained from the Lys-C generated peptide at *m/z* 530.2 from MRP-S29 is shown

Table 1
Amino acid sequences of peptides from pro-apoptotic proteins DAP3 and PDCD9 of *Bos taurus* derived from database searching or de novo sequencing* of peptide product ion spectra

Peptide #	Sequence	M_r (kDa)	p*I*
DAP3 peptides (MRP-S29)		43.6	8.75
Peptide 1	QDWLILHIPDAHLWVK		
Peptide 2	KSPLTPEELLLQ		
Peptide 3	SPNTPEELALLQ*		
Peptide 4	PLEAVTWLK		
Peptide 5	AYLPQELLGK		
Peptide 6	LQTSLNNDARQH		
Peptide 7	TVFPHGLPPRFVMQVK		
Peptide 8	NTNFAHPAVRYVLYGEK		
Peptide 9	HGEQHVGQHYNISIQELK		
PDCD9 peptides (MRP-S30)		48.5	6.83
Peptide 1	AAPVARYPPIVASLTADSK		
Peptide 2	YPPIVASLTADSK		
Peptide 3	NICWGTQSMPLYETIEDNDVK		

Whole 28S subunits prepared by two sequential sucrose gradients [9] were subjected to in situ endoprotease Lys C digestion to identify the mitochondrial small subunit ribosomal proteins in the intact subunit. Six of the peptides for DAP3 and all the peptides for PDCD9 shown were obtained by the latter method. Peptides 2, 3 and 4 for DAP3 were obtained from this protein following separation on 2D gels.

in Fig. 1. BLAST searches with the peptide sequence information obtained from initial Mascot searches indicated that nine peptides were derived from a protein identified as DAP3 or 'ionizing radiation resistance conferring protein' (U18321) (Table 1 and Fig. 1).

The human DAP3 ribosomal protein is 398 amino acids in length. Both MitoProt II and PSORT indicate that it has a 90% probability of being localized in mitochondria. The mature protein is predicted to have 380 residues and a molecular mass of 43.6 kDa. This value is in good agreement with the electrophoretic mobility of this protein. No known ribosomal proteins from other sources show significant similarities to the mammalian MRP-S29 (DAP3). Therefore, this protein represents a new class of ribosomal protein as are about half of the known mammalian mitochondrial ribosomal proteins [13]. Further analysis of this sequence suggests that DAP3 is a GTP binding protein. GTP binding proteins are characterized by three fairly well conserved motifs [24]. The first motif (the

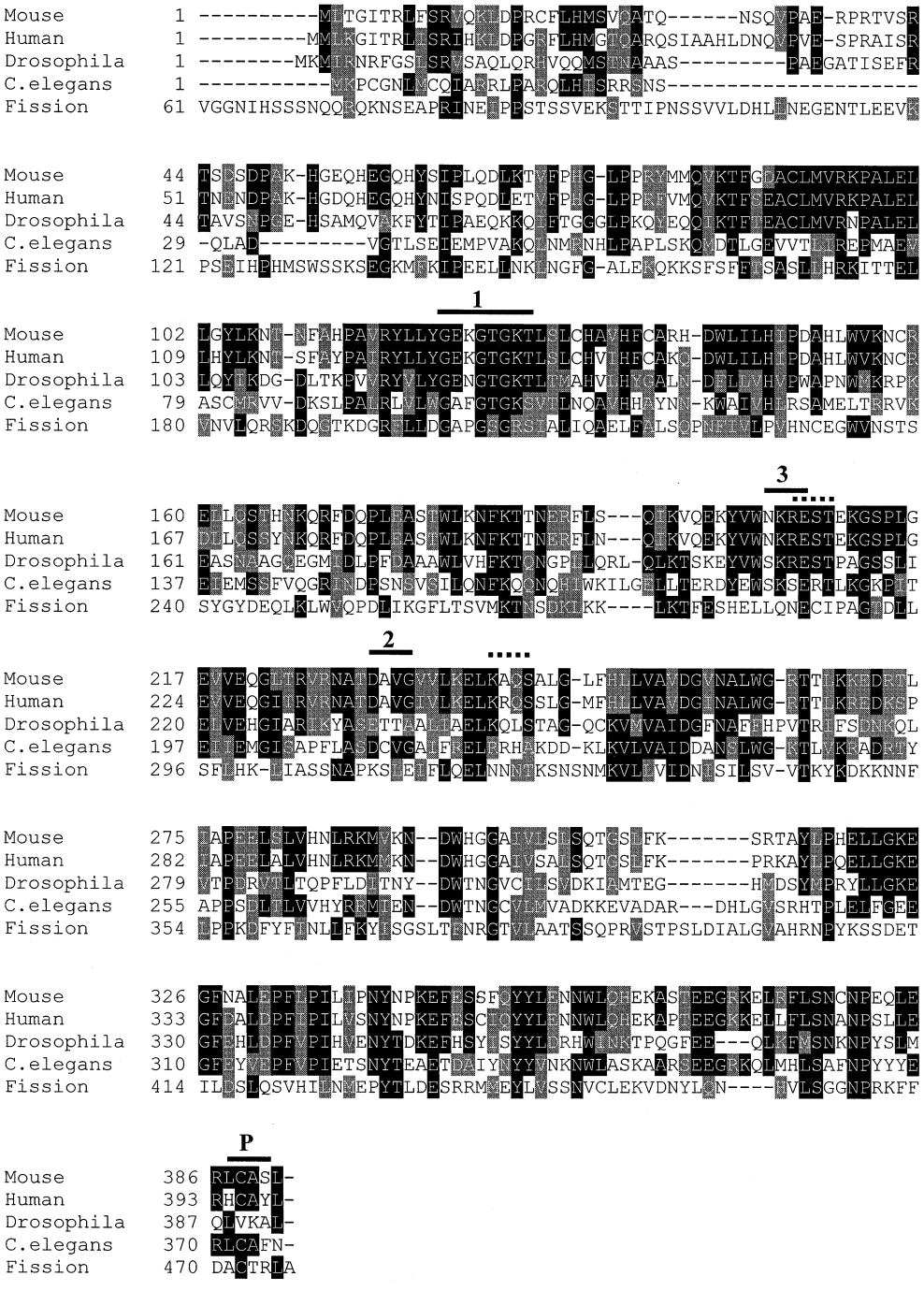

Fig. 2. Sequence of human DAP3 aligned to the corresponding proteins from mitochondria of other organisms. Fission indicates *S. pombe*. The sequence shown begins at position 61 for convenience. The three guanine nucleotide binding motifs are indicated by 1, 2 and 3. The predicted cAMP- and cGMP-dependent phosphorylation sites are indicated with thick dashed lines. The potential prenylation sequence is indicated with a P above the sequence.

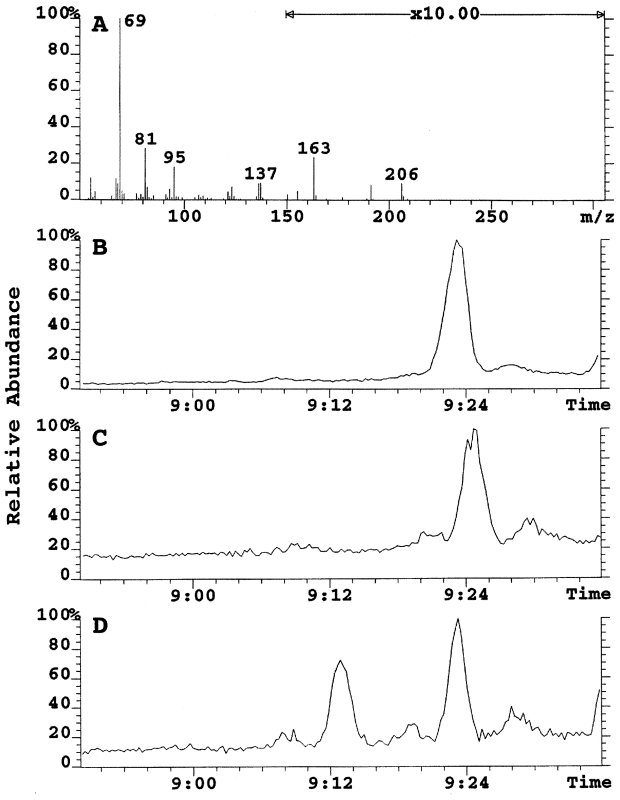

Fig. 3. Absence of prenylation of DAP3 in normal mitochondrial ribosomes. (A) Full fragmentation pattern of farnesene released from a control farnesylated tetrapeptide (farnesyl-*S*-Cys-Val-Ile-Ser-OH, Bachem AG) eluting at 9.13 min was analyzed by GC/MS in full scan mode and showed characteristic peaks at an *m/z* of 191.1800 and 206.2034. (B) Analysis of the presence of characteristic ions for farnesene (*m/z* 206.2034) in mitochondrial 55S ribosomes and (C) *E. coli* 70S ribosomes. (D) Determination of the detection limit of internally added farnesene in *E. coli* 70S ribosomes.

P-loop) is involved in binding the phosphate groups and has the consensus GXXXXGK(S/T) (Fig. 2). The second motif, normally seen as DXXG, is present as DAVG in DAP3. The third motif which determines the specificity for the guanine nucleotide (NKXD) is present as the conservative variant NKXE in DAP3. It should be noted that the usual order of these motifs has not been conserved in DAP3 since motif 2 is found following motif 3 in this protein. Interestingly, mammalian mitochondrial ribosomes are known to bind GDP and GTP tightly with a 1:1 stoichiometry [25]. DAP3 is the most likely candidate for the guanine nucleotide binding protein on these ribosomes since none of the other known sequences of small subunit ribosomal proteins (29 known to date) has a nucleotide binding site (Koc et al., in preparation). In addition to the GTP binding protein, DAP3, a putative guanine nucleotide exchange protein, MRP-S27, was identified among the mitochondrial small subunit proteins [14]. MRP-S27 might be a functional partner of DAP3. It is quite possible that nucleotide binding to DAP3 influences its interaction with the ribosome and with partner proteins that may mediate some event occurring during apoptosis. In addition to its GTP binding motif, DAP3 also possesses cAMP- and cGMP-dependent protein kinase phosphorylation sites (Fig. 2). The putative phosphorylation sites on DAP3 make this protein a potentially interesting target for key regulatory processes occurring during apoptosis.

Interestingly, the C-terminal tetrapeptide sequence of DAP3 (CAYL) resembles the consensus sequence motif specifying the prenylation of proteins. Prenylation occurs in a number of proteins including the heterotrimeric G-proteins involved in cell signaling events [26–28]. To examine the possibility that DAP3 might be prenylated in bovine mitochondrial ribosomes, purified mitochondrial 55S ribosomes were analyzed for either farnesyl or geranyl geranyl modifications using high resolution GC/MS and GC/MS/MS.

To establish conditions for the analysis of potential farnesyl groups on mitochondrial ribosomal proteins, a farnesylated peptide standard was treated with Raney nickel to release farnesene. The sample was analyzed by GC/MS in full scan mode using EI to provide a full fragmentation pattern. A peak eluting from the GC at 9.13 min gave the expected full scan fragmentation pattern for farnesene (Fig. 3A) showing characteristic peaks at an *m/z* of 191.1800 and 206.2034. To examine mitochondrial 55S ribosomes for prenylation, 1000 pmol of ribosomes were treated with Raney nickel and the resulting extract examined by GC/MS in SIM mode for the presence of peaks containing ions at an *m/z* of 191.1800 or 206.2034. The GC pattern showing elution of fractions with ions at *m/z* 206.2034 is shown in Fig. 3B. No peak was observed at 9.13 min where farnesene would appear if present. Similar experiments looking for ions with *m/z* 274.2661 ($M^{+}\bullet$) and 259.2426 indicated that these ribosomes were not modified with a geranyl geranyl group (data not shown). To examine the detection limits of this analysis, 1000 pmol of *Escherichia coli* ribosomes were treated in a similar manner and examined by GC/MS for the characteristic ion at *m/z* 206.2034. As expected (Fig. 3C) no peak with a retention time of 9.13 min was observed in this sample. This same *E. coli* ribosome sample was then spiked with 0.8 pmol of farnesene obtained from the Raney nickel-treated farnesylated tetrapeptide. Analysis of this sample clearly indicated the presence of a peak with the characteristic ion at *m/z* 206.2034 eluting at 9.13 min (Fig. 3D). Thus, prenylation occurring on less than 1% of the ribosomes would have been detectable. These results suggest that under normal metabolic conditions (bovine liver from healthy animals), DAP3 is not prenylated.

Homologs of DAP3 have been identified in other organisms (Table 2). The mouse homolog is over 80% identical to the human protein (Fig. 2 and Table 2). It contains all three of the motifs tentatively identified as GTP binding motifs as well as the putative C-terminal prenylation signal (Fig. 2). Both the *Drosophila melanogaster* and *Caenorhabditis elegans* homologs carry the P-loop motif for nucleotide binding. However, the putative motif 2 has been altered in the *D. melanogaster* and a variation of motif 3 is observed in the DAP3 from both species (Fig. 2). The third consensus element in

Table 2
Percentage identity of human MRP-S29 (DAP3) protein to mitochondrial homologs from other species

Source	Accession number	Length	Identity (%)
Mouse	–	391	81.8
Drosophila	CG3633	392	37.2
C. elegans	CAB60994	375	31.9
S. pombe	T40086	476	24.1
S. cerevisiae	Q01163	488	20.1

Table 3
Percentage identity of human MRP-S30 (PDCD9) protein to mitochondrial homologs from other species

Source	Accession number	Length	Identity (%)
Mouse	CAB90812[a]	441	74.8
Gallus gallus	AAC60367	465	62.1
D. melanogaster	AAF48515	557	28.1
C. elegans	AF067608	1023	27.8

[a]The mouse PDCD9 accession number is for the partial sequence, however the full length (441 amino acid) mouse protein was obtained by assembling EST sequences.

GTP binding proteins is often not as well conserved as motif 1. The mammalian and *C. elegans* homologs all contain the putative prenylation site. This site appears to be missing from *D. melanogaster*. However, if a sequencing error has caused an apparent frameshift very close to the C-terminus, a prenylation signal would be present in the *D. melanogaster* protein.

A *Schizosaccharomyces pombe* (pir accession number T40086) and a putative *Saccharomyces cerevisiae* homolog of DAP3 (SwissProt accession number Q01163, Ygl129c) were also detected in the databases (Fig. 2 and Table 2). The *S. pombe* and *S. cerevisiae* proteins have conserved the P-loop but do not contain either putative motif 2 or 3. Neither the *S. pombe* nor the *S. cerevisiae* proteins has a prenylation signal at the C-terminus.

3.2. PDCD9 (p52)

Whole proteolytic digestions of 28S and 39S subunits followed by LC/MS/MS and Mascot database searching matched three peptide sequences (Table 1) derived from the proapoptotic protein PDCD9, also known as p52 (GenBank accession number NP_057724) [5,6]. Although this protein was found in preparations of both 28S and 39S subunits, we believe that it is present in the 28S subunit since these preparations were largely free of contamination by the large subunit while the 39S preparation did have some cross-contamination with small subunits.

Homologs of PDCD9 (now designated MRP-S30) are present in other eukaryotes although it is not highly conserved (Table 3). No homologs could be detected in *S. pombe* or *S. cerevisiae* although the low conservation of the sequence of this protein might make it difficult to detect. Protein database searches do not reveal any significant similarities to other known proteins with the exception of a very weak similarity to several chloroplast S4 ribosomal proteins (less than 20% identity). However, residues generally conserved in the S4 proteins found in a variety of prokaryotes, chloroplasts and in yeast mitochondria are not conserved in PDCD9 suggesting that it is not a mammalian mitochondrial homolog of bacterial S4.

The results presented here support the notion that mitochondria play a central role in many of the major cellular apoptotic pathways. During apoptosis, a number of proteins are exported from mitochondria while a number of others are associated with this organelle. It is possible that either or both DAP3 and PDCD9 are released from the ribosome and exported from mitochondria during apoptosis. Alternatively, they may play a role in mitochondrial events such as changes in the permeability pore transition that occur during apoptosis. The association of a significant portion of mitochondrial ribosomes with the inner membrane makes this a reasonable possibility [29].

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References

[1] Brenner, C. and Kroemer, G. (2000) Science 289, 1150–1151.
[2] Gottlieb, R.A. (2000) FEBS Lett. 482, 6–12.
[3] Bernardi, P., Scorrano, L., Colonna, R., Petronilli, V. and Di Lisa, F. (1999) Eur. J. Biochem. 264, 687–701.
[4] Crompton, M. (1999) Biochem. J. 341, 233–249.
[5] Sun, L., Liu, Y., Fremont, M., Schwarz, S., Siegmann, M., Matthies, R. and Jost, J.P. (1998) Gene 208, 157–166.
[6] Carim, L., Sumoy, L., Nadal, M., Estivill, X. and Escarceller, M. (1999) Cytogenet. Cell Genet. 87, 85–88.
[7] Kissil, J.L., Cohen, O., Raveh, T. and Kimchi, A. (1999) EMBO J. 18, 353–362.
[8] Kissil, J.L., Deiss, L.P., Bayewitch, M., Raveh, T., Khaspekov, G. and Kimchi, A. (1995) J. Biol. Chem. 270, 27932–27936.
[9] Matthews, D.E., Hessler, R.A., Denslow, N.D., Edwards, J.S. and O'Brien, T.W. (1982) J. Biol. Chem. 257, 8788–8794.
[10] Cahill, A., Baio, D. and Cunningham, C. (1995) Anal. Biochem. 232, 47–55.
[11] Koc, E.C., Blackburn, K., Burkhart, W. and Spremulli, L.L. (1999) Biochem. Biophys. Res. Commun. 266, 141–146.
[12] Shevchenko, A., Wilm, M., Vorm, O. and Mann, M. (1996) Anal. Chem. 68, 850–858.
[13] Koc, E.C., Burkhart, W., Blackburn, K., Moseley, A., Koc, H. and Spremulli, L.L. (2000) J. Biol. Chem. 275, 32585–32591.
[14] Koc, E.C., Burkhart, W., Blackburn, K., Koc, H., Moseley, A. and Spremulli, L.L. (2000) Protein Sci., in press.
[15] Burkhart, W. (1992) in: Techniques in Protein Chemistry IV (Angeletti, R., Ed.), pp. 399–406, Academic Press, New York.
[16] Perkins, D.N., Pappin, D.J., Creasy, D.M. and Cotrell, J.S. (1999) Electrophoresis 20, 3551–3567.
[17] Pearson, W.R. and Lipman, D.J. (1988) Proc. Natl. Acad. Sci. USA 85, 2444–2448.
[18] Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D.J. (1997) Nucleic Acids Res. 25, 3389–3402.
[19] Nakai, K. and Kanehisa, M. (1992) Genomics 14, 897–911.
[20] Claros, M.G. and Vincens, P. (1996) Eur. J. Biochem. 241, 770–786.
[21] Ham, A.J., Ranasinghe, A., Koc, H. and Swenberg, J.A. (2000) Chem. Res. Tox. 13, 1243–1250.
[22] Brown, H.C. and Krishnamurthy, S. (1973) J. Am. Chem. Soc. 95, 1669–1671.
[23] Corey, E.J., Kim, C.U. and Takeda, M. (1972) Tetrahedron Lett. 42, 4339–4342.
[24] Kjeldgaard, M., Nyborg, J. and Clark, B.F.C. (1996) FASEB J. 10, 1347–1368.
[25] Denslow, N., Anders, J. and O'Brien, T. (1991) J. Biol. Chem. 266, 9586–9590.
[26] Casey, P. (1995) Science 268, 221–225.
[27] Zhang, F. and Casey, P. (1996) Annu. Rev. Biochem. 65, 241–269.
[28] Overmeyer, J.H., Erdman, R.A. and Maltese, W.A. (1998) Methods Mol. Biol. 88, 249–263.
[29] Liu, M. and Spremulli, L.L. (2000) J. Biol. Chem. 275, 29400–29406.