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Evidence for a second messenger function of dUTP during Bax mediated apoptosis of yeast and mammalian cells

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ABSTRACT

The identification of novel anti-apoptotic sequences has lead to new insights into the mechanisms involved in regulating different forms of programmed cell death. For example, the anti-apoptotic function of free radical scavenging proteins supports the pro-apoptotic function of Reactive Oxygen Species (ROS). Using yeast as a model of eukaryotic mitochondrial apoptosis, we show that a cDNA corresponding to the mitochondrial variant of the human DUT gene (DUT-M) encoding the deoxyuridine triphosphatase (dUTPase) enzyme can prevent apoptosis in yeast in response to internal (Bax expression) and to exogenous (H_2O_2 and cadmium) stresses. Of interest, cell death was not prevented under culture conditions modeling chronological aging, suggesting that DUT-M only protects dividing cells. The anti-apoptotic function of DUT-M was confirmed by demonstrating that an increase in dUTPase protein levels is sufficient to confer increased resistance to H_2O_2 in cultured C2C12 mouse skeletal myoblasts. Given that the function of dUTPase is to decrease the levels of dUTP, our results strongly support an emerging role for dUTP as a pro-apoptotic second messenger in the same vein as ROS and ceramide.

1. Introduction

Apoptotic cell death (type I) is a genetically programmed mechanism that allows the cell to commit suicide when conditions are judged to be unfavorable [1]. Although the term apoptosis is often used to denote all forms of programmed cell death (PCD), it should be noted that genetically encoded cell death can occur via a number of different but likely interrelated mechanisms such as autophagy (type II PCD) and necrosis (type III PCD) [2]. The decision on whether a cell commits suicide in response to a given stress is dependent on the interplay of a number of regulatory pro- and anti-apoptotic proteins [3]. For example, Bax, the most well studied pro-apoptotic member of the Bcl-2 family of proteins, functions, at least in part, by interacting with the mitochondrial membrane and assisting the release of pro-apoptotic molecules such as cytochrome c. Other proteins like caspases become activated by stress responsive cascades and they serve to promote cell death by cleaving a variety of cellular substrates that lead to death [4]. In contrast, many of the anti-apoptotic proteins function by directly antagonizing apoptotic proteins. For example, the anti-apoptotic Bcl-2 is probably the most studied inhibitor of apoptosis. It functions in part, by opposing the effects of pro-apoptotic Bcl-2 members such as Bax [5]. Other anti-apoptotic proteins include the structurally diverse group of heat shock proteins (HSPs), a variety of antioxidant proteins as well as a number of other proteins of unknown function such as the GTPase GIMAP8 [6,7]. In spite of recent advances, more is known about the processes involved in initiating death than in the processes involved in preventing cell death [8,9]. One factor that likely serves to limit our knowledge of anti-apoptosis is the fact that the complete repertoire of cell survival proteins is not known.

A number of different approaches have been successful in identifying novel anti-apoptotic genes. One successful strategy is to characterize genes that are up-regulated in a variety of different conditions where cells have increased resistance to apoptosis. A number of anti-apoptotic genes have thus been identified from commonly described apoptotic resistant cells including cancer cells, cells treated with sublethal levels of stresses (i.e. pre-conditioning) and neuronal cells subjected to increased stimulation [10–14]. The disadvantage here is the large number of genes that are up-regulated in such cells and the lack of knowledge regarding their potential as anti-apoptotic sequences. Another widely used strategy involves the screening of heterologous cDNA expression libraries in yeast

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ectopically expressing a mammalian cDNA corresponding to the pro-apoptotic Bax [15]. Such screens have the advantage of yielding sequences that have pro-survival functions. Such an approach has been validated by the fact that the characterization of numerous sequences identified as Bax suppressors in yeast have been shown to be anti-apoptotic when overexpressed in mammalian cells [16–18]. This is not surprising given that many different stresses including ROS donors and ectopically expressed Bax leads to Programmed Cell Death in yeast that shares a great deal of similarities with mammalian mitochondrial centered apoptosis [15]. The similarity is such that yeast is now a widely used model for apoptosis and other aspects of PCD [19–21]. In addition, this functional interchangeability of yeast and mammalian genes, has for many years lead to the use of so-called "humanized yeast" as a useful model to study the structure and function of heterologously expressed mammalian genes [18]. We have previously carried out Bax suppressor screens and have described the isolation of several potential mammalian anti-apoptotic sequences [22]. Here, we report that one of these uncharacterized clones encodes a cDNA corresponding to mitochondrially targeted dUTPase protein encoded by the human DUT gene. We further show that overexpression of the dUTPase cDNA renders yeast and mammalian cells resistant to stress induced cell death. Our results provide compelling evidence that serves to support the notion that dUTP is a second messenger that mediates the pro-apoptotic effects of cellular stresses in growing cells.

2. Materials and methods

2.1. Yeast strains and plasmids

The *Saccharomyces cerevisiae* BY4741 (*MAT***a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0) strain was used throughout this study (EUROSCARF). The DUT-M and HSP90β clones, expressed under the control of the galactose inducible *GAL1* promoter in pYES-DEST52, was isolated in our previous screen of a human cardiac cDNA library for Bax suppressors [22]. Of the multitude of different clones isolated in the screen, DUT-M was the only cDNA that was isolated three independent times.

2.2. Yeast transformations, growth and viability assays

Yeast were grown in synthetic minimal media containing yeast nitrogen base (YNB), 2% glucose and supplemented with the required amino acids (aa) or bases. When expression of the *GAL1* promoter was required, the glucose was substituted with 2% galactose and 2% raffinose. Yeast cells were transformed with the appropriate plasmids using the lithium acetate method and transformants were selected for by the omission of the appropriate nutrient (uracil for pYES-DEST52 plasmids). The rich media used consisted of 2% bactopeptone, 1% yeast extract and 2% glucose (YEPD). The clonogenecity assay was used to determine viability. Briefly, freshly saturated overnight cultures of the different yeast transformants were diluted in fresh, pre-warmed galactose-containing media, incubated for 4 h to induce gene expression, and subsequently treated with the indicated concentration of H_2O_2 or cadmium. Aliquots were harvested, serially diluted and triplicate samples of 300 cells were then plated on YEPD media, grown at 30 °C and the number of colonies that formed after 2 days were counted. The same procedure was used to generate chronologically aged cultures except that the cells were continuously incubated for 14 days and samples were harvested daily to determine viability. Alternatively, viability was also determined by microscopic examination of cells stained with the vital dye trypan blue. Cells were stained with 0.1% trypan blue for 5 min and at least 300 cells were scored for each time point.

2.3. C2C12 cell culture

Mouse C2C12 myoblasts were cultured at 37 °C in a controlled incubator of 5% CO_2, maintained in growth medium containing DMEM (Dulbecco's modified Eagle's medium) and supplemented with 10% fetal bovine serum and antibiotics (streptomycin and penicillin) [23]. Cells were infected in 100-mm tissue culture plates at ≈80% confluency with HSV (herpes simplex virus) infectious stock to allow for 1:150 dilution of virus:growth medium. Cells were then incubated in 37 °C CO_2 incubator for 4 h, after which cell culture plates were washed 3 times with PBS and replaced with growth medium. Cells were then incubated for 24 h in a 37 °C CO_2 incubator. Oxidative stress was stimulated by exposing cells to different concentrations of H_2O_2 in DMEM without serum or antibiotics for 24 h in a 37 °C CO_2 incubator. Following H_2O_2 exposure, cells were pelleted by centrifugation at 3000 rpm for 3 min and analyzed by microscopy following staining with DAPI or via APO-BrdU TUNEL assay (Invitrogen).

2.4. Expression of Recombinant HSV constructs

The cDNAs corresponding to the entire open reading frame of DUT-M and HSP90β were individually inserted into an amplicon plasmid pHSV-PrpUC, containing the immediate early promoter 4/5 of HSV, and an HSV-packaging site [24]. The plasmid (2 μg) was subsequently transfected into the HSV packaging cell line 2-2 (3×10^5) with lipofectamine (12 μl) and then infected with 5 dl5 HSV helper virus after 24 h after incubation. The recombinant virus was amplified through three sequential rounds of infection and then stored at -80 °C.

2.5. APO-BrdU TUNEL and flow cytometry analysis

Cells were grown in 100-mm tissue culture plates until ≈80% confluent, infected with HSV-constructs, and exposed to H_2O_2 as described above. Apoptotic cells were detected by the terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL) staining using the fluorescence method with the APO-BrdU TUNEL assay kit (Invitrogen) according to manufacturer's instructions. Following TUNEL protocol, cells were immediately analyzed in a FACScan (Becton Dickinson). The percentage of apoptotic positive cells was performed with Cell Fit software (Becton Dickinson).

2.6. Protein extraction and western blot analysis

Soluble protein was extracted from cultured cells using 1 ml of ice-cold lysis buffer per dish (50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 0.1% SDS, 100 μg/ml PMSF, and 1% NP-40). Twenty μg of soluble proteins were separated by SDS-PAGE, transferred to nitrocellulose membrane and incubated with different primary anti-serum [25]. Commercially available rabbit anti-dUTPase, HSP90 and of α-tubulin from Santa-Cruz Biotechnology (Santa Cruz, California) were used as described by the manufacturer. HRP-conjugated secondary anti-serum was used and signals were subsequently detected with ECL plus (Amersham Bioscience) followed by exposure to X-ray film (Kodak X-Omat).

3. Results

Our lab previously isolated multiple mammalian cDNAs capable of suppressing the negative effects of Bax in yeast [22]. We have since characterized four of these Bax suppressors and have determined that they are anti-apoptotic sequences since they are capable of preventing or delaying cell death in response to a variety of other pro-apoptotic stimuli in yeast [15,22,26,27]. Here, we report the characterization of another Bax suppressor corresponding to a human cDNA encoding a predicted 252 residue protein. Analysis of the sequence by comparing