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DUG is a novel homologue of translation initiation factor 4G that binds eIF4A

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Abstract

To elucidate the molecular mechanisms of cell death, we have cloned a new gene, designated death-upregulated gene (DUG), from rat insulinoma cells. DUG is constitutively expressed at very low levels in normal cells but is dramatically upregulated in apoptotic cells following serum/glucose starvation or death receptor ligation by Fas ligand. The DUG mRNA is present in two splicing forms: a long form that encodes a protein of 469 amino acids and a short form that gives rise to a polypeptide of 432 amino acids. The predicted DUG protein sequence contains two putative nuclear localization signals and multiple phosphorylation sites for protein kinases and two conserved MA3 domains. Importantly, DUG is homologous to eukaryotic translation initiation factor (eIF) 4G and binds to eIF4A presumably through MA3 domains. Upon transfection, DUG inhibits both intrinsic and extrinsic pathways of apoptosis. Thus, DUG is a novel homologue of eIF4G that regulates apoptosis.

Keywords: Apoptosis; Protein synthesis; eIF4G homologue; MA3 domain; pdcd4

Programmed cell death, or *apoptosis*, is responsible for a significant proportion of cell death in many bioprocesses including embryogenesis, metamorphosis, endocrine-dependent tissue atrophy, and normal tissue turnover as well as immune homeostasis [1–6]. Apoptosis consists of cascades of events that involve various genes, enzymes, and signalling pathways. The cascades may be divided into two different phases: the initiation phase and the effector phase [2–4]. During the initiation phase, the inducers of apoptosis, which include a variety of physical, chemical, or biological agents, interact with the target molecules in the cell and initiate various apoptotic cascades. Once the apoptotic cascades are initiated, downstream effector molecules, such as caspases, will be recruited, which in turn lead to disassembly of the cells [3,7]. The initiation phase of apoptosis may include a number of parallel pathways, which converge upon one, or at most a few, pathways of the effector phase. Identification of molecules involved in the inductive and effector phases of apoptosis is crucial for our understanding of the molecular mechanisms of cell death and its regulation.

The initiation of protein synthesis is a complex process, which involves a large number of initiation factors and RNA molecules. An early event during the initiation process is the formation of the ternary complex consisting of eukaryotic initiation factor (eIF) 2, methionyl-tRNA, and GTP, which binds to the 40S ribosomal subunit [8]. eIF4G is a key molecule for translation initiation, as it serves as a bridge for other proteins of the ribosomal initiation complex. Together with the cap-binding protein eIF4E and the ATP-dependent RNA helicase eIF4A, eIF4G forms the initiation factor eIF4F [9]. Additionally, eIF4G contains binding domains for the eIF3 complex, the poly(a) binding protein, and the eIF4E kinase Mnk1 [10–13].

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The interaction of eIF4G with eIF4E enhances the binding to the 5′ mRNA cap. This binding is inhibited by members of the 4E-binding protein (4E-BP) family. Phosphorylation of eIF4E by the eIF4E kinase Mnk1 results in an enhanced translational activity. Induction of apoptosis can lead to significant inhibition of protein synthesis, especially during the later stages of apoptosis. This may relate to the caspase-dependent cleavages of initiation factors eIF4G, eIF4B, and eIF2α [14–17], the enhanced binding of 4E-BP to eIF4E [3,15], and the phosphorylation of eIF2α [18], which inhibits the formation of the ternary complex. However, the roles of translation factors in regulating apoptosis are not well established.

To identify new genes that are involved in regulating apoptosis, we performed differential display analysis of rat insulinoma cells that had been treated under normal or apoptotic conditions. We identified several new genes that were either up- or downregulated during apoptosis. One of these new genes, designated as death-upregulated gene (DUG), is a homologue of the eukaryotic initiation factor 4-G. We report here that DUG binds to eIF4A and inhibits both intrinsic and extrinsic pathways of apoptosis.

Materials and methods

Cell culture. Rat insulinoma (INS-1) cells were cultured in RPMI 1640 medium supplemented with 10% foetal calf serum, 1% penicillin/streptomycin, and 0.05 mM 2-β-mercaptoethanol. Rat-1 and HEK293 cells were maintained in DMEM containing 10% foetal calf serum and 1% penicillin/streptomycin.

Cloning of DUG. Rat insulinoma cells (INS-1) were incubated for 5 days in the presence of 1, 5, 11, 20, and 30 mM glucose. Total RNA was isolated using the RNeasy Kit (Quiagen). Differential display was performed with the RNAimage Kit (Gene Hunter, Nashville, TN) according to manufacturer's instructions. cDNA fragments obtained were amplified by PCR and sequenced. Nucleotide sequences were used for homology searches of the available databases. The specific 5′ end of DUG was cloned using the First Choice RLM-RACE Kit (Ambion, Austin, TX) according to manufacturer's instructions. The sequence of the inner gene-specific primer was 5′-CGCAGTCGT CTTTTTGCTTTAGC-3′; the sequence of the outer gene-specific primer was 5′-GGATGAATTTTTCCGCAGTCG. Full-length DUG was obtained by PCR with an antisense primer homologous to the 3′ region and a sense primer homologous to the 5′ region of DUG with total RNA from INS-1 cells as template (GenBank Accession No. AF239739).

Northern blot analyses. For Northern blot analyses of DUG, blots were hybridized with a ^{32}P-labelled cDNA fragment obtained by PCR using the sense primer GAAGAAAATGCTGGCACTGAGG and the anti-sense primer TCGGGGAGATCCTTCAACAAC and full-length DUG as template. The blots were washed under stringent conditions (0.1× SSC, 0.1% SDS) at 68 °C and exposed to X-ray film at −70 °C with two intensifying screens for up to 6 h.

Production of recombinant adenoviruses carrying FasL. Recombinant adenovirus carrying the mouse FasL gene was generated as we described previously [19].

Generation of DUG-expressing cell lines. Full-length DUG cDNA was cloned by RT-PCR using total RNA from INS-1 cells and the primers GGCGAATTCACCATGGATATAGAAAATGAGCAGA TA and GGCTCTAGAATGTAGCTCTCAGGTTTAAGACGA CC, which contained an *Eco*RI and a *Xba*I site, respectively. The *Eco*RI/*Xba*I fragment was inserted into the corresponding sites of pcDNA3.1-V5-His generating pcDNA3.1-DUG-V5-His. HEK293 cells were transfected with pcDNA3.1-DUG-V5-His using the calcium precipitation method according to established protocols. Controls were mock-transfected with pcDNA3.1-V5-His. Selection of transfected cells was performed with geneticin (G418). The expression of DUG was confirmed by Western blot using an HRP-coupled anti-V5-antibody (invitrogen, Karlsruhe, Germany).

Apoptosis assay. Cells were grown in 24-well cell culture plates and incubated in the absence or presence of TRAIL (200 ng/ml), cycloheximide (2 μg/ml) or bisphenol A diglycidyl ether (BADGE; 80 μM) for 16 h at 37 °C. Cells were then harvested, washed, and resuspended in ice-cold PBS (pH 7.4) containing 1 mM glucose and 2 μg/ml propidium iodide. PI fluorescence intensity was determined by flow cytometry using the FACScan flow cytometer. Data analysis was performed using the Cell Quest software program (Becton–Dickinson, San Jose, CA).

Immunoprecipitation. DUG-expressing cells were co-transfected with pcDNA 3-FLAG-eIF4A (4 μg plasmid; provided by Drs. Imataka and Sonenberg) using Lipofectin (Gibco-BRL, Rockville, MD) according to manufacturer's protocol. After 48 h, cells were treated with ice-cold lysis buffer (Tris–HCl 10 mM, pH 7.4; EDTA 1 mM, and PMSF 1 mM) and sonicated. Cell debris was removed by centrifugation and supernatant was incubated with anti-V5-antibody (8 μg/ml) for 16 h at 4 °C. Protein A/G agarose (Oncogene, Boston, MA) was then added to the sample and incubation continued for an additional hour. After washing three times, beads were resuspended in electrophoresis loading buffer and heated for 5 min at 95 °C. Equal amounts of protein were then loaded onto a polyacrylamide gel and fractionated by electrophoresis. After transfer to a nitrocellulose membrane, eIF4A was visualized using an HRP-coupled anti-FLAG-M2-antibody (Sigma, Taufkirchen, Germany).

Results

DUG expression is dramatically upregulated under apoptotic conditions

To isolate new genes that are upregulated during apoptosis, we first cultured rat INS-1 cells for 5 days under either low (1–11 mM) or high (30 mM) glucose condition that induces apoptosis. Using the differential display technique, we identified a cDNA, later named DUG (death upregulated gene), which was upregulated under both low and high glucose conditions. The normal glucose concentration in the culture, which was 20 mM, was used as the control.

To determine whether upregulation of DUG also occurs in other cell types, we tested DUG expression in human 293 and mouse NIH3T3 cells under conditions that deprive them of all nutrients. Thus, 293 and NIH3T3 cells were cultured at 37 °C in PBS, in the total absence of glucose and serum, for up to 24 h and their mRNA was extracted and blotted with DUG cDNA probe. As shown in Fig. 1, DUG expression was barely detectable in cells under normal culture condition, but was dramatically upregulated upon starvation. The upregulation of DUG occurred almost immediately

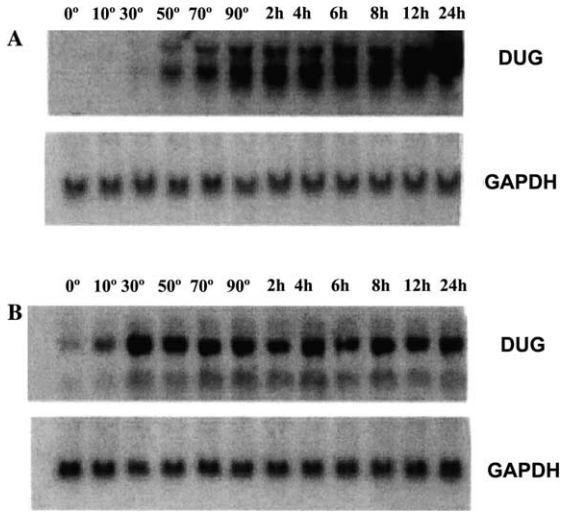

Fig. 1. Kinetics of DUG upregulation in 293 and NIH3T3 cells in response to serum and glucose starvation. Apoptosis was induced by incubation of (A) 293 or (B) NIH3T3 cells in phosphate-buffered saline (PBS). At the indicated times, total RNA was prepared and DUG mRNA expression was determined by Northern blot analysis. Each lane contains 10 μg total RNA. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a reference.

(10–30 min) following starvation and reached a plateau 30–90 min later.

To determine whether other apoptotic inducers also upregulate DUG expression, we studied FasL-induced apoptosis in 293 cells. Thus, 293 cells were infected with 10^{10} particles of a recombinant adenovirus carrying the proapoptotic FasL gene. Control cells were infected with an empty viral vector Ad-BglII. DUG was rapidly upregulated in cells infected with Ad-FasL but not in cells infected with Ad-BglII, suggesting that DUG expression is also regulated by FasL-induced apoptotic pathway.

DUG is a homologue of the translation factor eIF4-G

The cDNA fragment of DUG, identified in the differential display, was amplified by PCR and sequenced. A homology search of the available databases revealed that the nucleotide sequence of DUG was highly homologous to the 3′ sequences of the mouse MA-3 and TIS genes [20,21] and the human genes H731 and 197/15a [22,23], respectively. After cloning the 5′ end of the coding region, the full-length nucleotide sequence of DUG was obtained by PCR using specific 3′ and 5′ primers with total RNA from INS-1 cells as template. Analysis of the PCR products by agarose gel electrophoresis showed two specific bands. Sequencing analysis revealed that the two fragments shared a common sequence of ~1 kb, with the larger one having an additional 112 bp. The larger PCR fragment contained an open reading frame coding for a protein of 469 amino acids. The smaller fragment contained an open reading frame coding for a protein lacking 37 amino acids (amino acids 367–403) of the larger protein. Screening of the available databases using advanced BLAST search showed significant alignments of the amino acid sequences of mouse MA-3 and TIS genes as well as the human H731 and 197/15a genes. Therefore, DUG may represent the rat homologue of the aforementioned mouse and human genes. Further analysis of the primary protein sequence revealed the presence of two putative nuclear localization signals (^{58}KAKRRLR^{64} and ^{484}PSRGRKR^{454}) as well as multiple putative phosphorylation sites for kasein kinase II, proline-directed protein kinase, and protein kinase C (Fig. 2).

Importantly, sequence alignment analysis also revealed that DUG shares significant amino acid homology with the eIF4G family of proteins. It has 23–29% identity and 46–52% similarity with the middle region of eIF4G from various species. Additionally, DUG contains conserved alpha helical MA3 domains (amino acids 164–275 and 329–440) (Fig. 2), which are also

MDIENEQILNVNPTDPDNLSDSLFSGDEENAGTEEIKNEINGNWISASTINEARINA

*KAKRRLR*KNSSRDSGRGDSVSDNGSEAVRSGVAVPTCPKGRLLDRRSRSGKGR

GLPKKGGAGGKGVWGTPGQVYDVEKVDVKDPNYDDDQENCVYETVVLPLDET

AFEKTLTPIIQEYFEHGDTNEVAEMLRDLNLWEMKSGVPVLAVSL

ALEGKASHREMTSKLLSDLCGTVMSTNDVEKSFDKLLKDLPELA

LDTPRAPQLVGQFIARAVGDGILCNTYIDSYKGTVDCVQARAALDKATVL

LSMSKGGKRKDSVWGSGGGQQACHPLVKEIDMLLKEYLLSGDMSEAEH

CLKELKVPHFHHELVYEAIVMVLESTGESAFKMMLGLLKSLWKS

STITIDQMKRGYERIYNEIPDINLDVPHSYSVLERFVEECFQAGIISK

QLRDLC*PSRGRKR*FVSEGDGGRLKPESY*

Fig. 2. Analysis of the amino acid sequence of DUG. Amino acids not present in the splice variant are underlined. The putative nuclear localization signals are shown in italics. The conserved MA3 domains are shown in bold. Putative proline-directed protein kinase phosphorylation sites (S/T P) are in shadowed boxes. Putative casein kinase II phosphorylation sites (T/S XX D/E) are shown with dark background. Putative protein kinase C phosphorylation sites (X (R/K_{1-3}, X_{2-0}) (S/T) (X_{2-0}, R/K_{1-3}) X) are boxed.

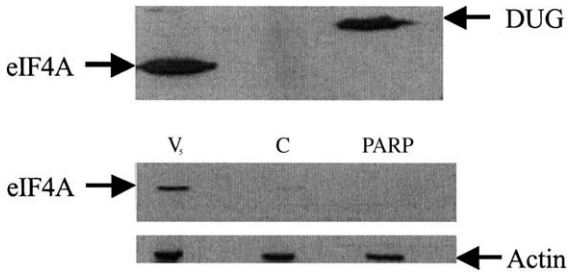

Fig. 3. DUG binds eukaryotic initiation factor 4A. Upper panel: DUG-V5-His expressing cells were co-transfected with a FLAG-eIF4A plasmid. Cell lysates were analyzed by immunoblotting with anti-V5-antibody and anti-FLAG-M2-antibody, demonstrating the presence of DUG and eIF4A, respectively. Lower panel: DUG-V5-His expressing cells were cotransfected with a FLAG-eIF4A plasmid. Cell lysates were immunoprecipitated with anti-V5-antibody (V5; IgG2a) and analyzed by immunoblotting with anti-FLAG-M2-antibody. Controls were either not treated with the anti-V5-antibody (C) or treated with an unrelated IgG2a antibody (PARP). Results shown are representative of two separate experiments.

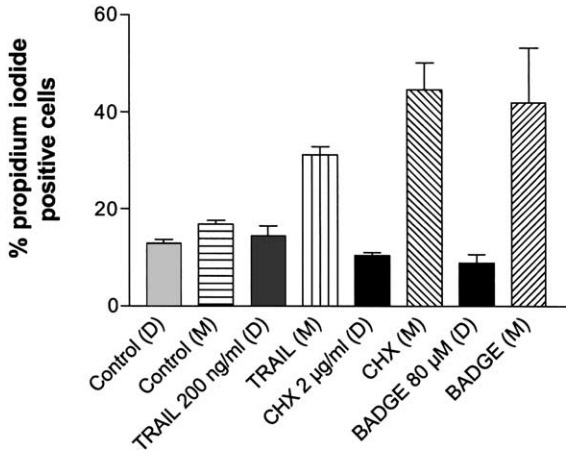

Fig. 4. DUG expressing cells are protected against apoptosis. DUG (D) or mock (M)-transfected cells were incubated with or without TRAIL (200 ng/ml), cycloheximide (CHX, 2 μg/ml) or BADGE (80 μM) for 16 h at 37 °C. After staining with propidium iodide, cells were analyzed for apoptosis by flow cytometry. Data shown are means ± SEM of duplicate cultures. The experiment was repeated three times with similar results.

present in eukaryotic translation initiation factors, eIF4G I and eIF4G II. The MA3 domain is involved in protein–protein interactions and enables eIF4G to bind to eIF4A [24].

DUG interacts with eukaryotic initiation factor-4A

To explore the biological functions of DUG, we first transfected HEK293 cells with a DUG pcDNA3.1 plasmid carrying a V5 and a histidine tag. After selection with G418, Western blot analysis was performed using an anti-V5-antibody to demonstrate DUG protein expression (Fig. 3).

To determine whether DUG could interact with eIF4A, DUG expressing cells were co-transfected with pcDNA3-FLAG-eIF4A. Forty hours later, cell extracts were prepared and immunoprecipitation was performed. Precipitated proteins were separated by PAGE and FLAG-eIF4A was visualized using an HRP-coupled anti-FLAG-M2 antibody. As shown in Fig. 3, DUG-expressing cells also expressed FLAG-eIF4A. When immunoprecipitation was performed using an anti-V5-antibody (IgG2a) directed against the fusion protein DUG-V5-His, FLAG-eIF4A was found to be co-immunoprecipitated (Fig. 3). If the experiments were performed without anti-V5 antibody or in the presence of a control IgG2a antibody (for example, an antibody against PARP), no FLAG-eIF4A band was detected. These results indicate that DUG can bind to eIF4A presumably via the conserved MA3 domains.

DUG-expressing cells are resistant to apoptosis

Since DUG is upregulated in apoptosing cells, we investigated whether DUG expression would alter the basal level of apoptosis or apoptosis induced by various stimuli. Thus, mock-transfected cells and DUG-transfected cells were cultured with or without TNF-related apoptosis inducing ligand (TRAIL) [25], cycloheximide [26], or bisphenol A diglycidyl ether (BADGE) [27]. These substances have been shown to induce apoptosis via mitochondria-dependent (intrinsic) or mitochondria-independent (extrinsic) pathways. As shown in Fig. 4, increased DUG expression did not induce apoptosis, but significantly abrogated the apoptosis induced by all the apoptotic stimuli. Taken together, these results suggest that DUG may protect cells from both intrinsic and extrinsic pathways of apoptosis.

Discussion

In an attempt to isolate cDNA fragments that are upregulated during the induction of apoptosis, we have cloned a novel gene called DUG. DUG encodes a putative protein of 469 amino acids and a shorter variant lacking 37 amino acids. Homology search of the available databases revealed that DUG is the rat homologue of mouse MA-3 and TIS genes (also termed pdcd4) [20,21] and human H731 and 197/15a genes [21,22]. Using mouse MA-3 cDNA as a probe, Shibahara et al. [20] detected cross-hybridizing mRNA in human, chicken, and *Xenopus* and *Drosophila* tissues, demonstrating a high degree of conservation of this gene during evolution. However, although it has been recently shown that MA-3 inhibits tumour promoter-induced neoplastic transformation [28], the biological roles of MA-3 and its homologues are not clear.

Apoptosis may be initiated through a number of parallel pathways that converge upon one, or at most a

few, effector pathways. Upregulation of DUG is an early event during apoptosis, since increased DUG expression was observed 10 min after the induction of cell death. Because many different apoptotic conditions, such as starvation, high glucose levels, or activation of death receptors by FasL, can upregulate DUG, downstream apoptotic signals are likely involved in regulating DUG gene expression.

DUG contains putative nuclear localization signals in both its N- and C-termini. It also has several putative phosphorylation sites of protein kinases. These suggest that DUG can traffic between nucleus and cytoplasm, and that its function can be regulated by phosphorylation. Consistent with this theory, DUG homologues have been detected in both cytoplasm and nucleus in HeLa and JB6 cells [28].

Interestingly, at amino acid positions 164–275 and 329–440, DUG contains two conserved α-helical MA3 domains. Such domains are also present in eukaryotic translation initiation factors 4G-I and 4G-II. The MA3 domain is involved in protein–protein interactions and enables eIF4G to bind eIF4A [24]. Together with eIF4E and eIF4G, eIF4A forms the eIF4F complex. While eIF4G serves as a bridge for initiation factors eIF4A and eIF4E, eIF4E binds to the 5′ mRNA cap structure and eIF4A acts as an ATP-dependent RNA helicase, which unwinds the mRNA allowing the ribosome to scan along the mRNA in a 3′ direction to locate the initiating AUG codon. We found that DUG, which is rapidly upregulated after apoptosis induction, interacts with eIF4A. However, unlike eIF4G, DUG does not bind to IF-3 (unpublished data). Since induction of apoptosis can lead to rapid and substantial inhibition of protein synthesis, it is possible that DUG may inhibit translation by blocking the formation of eIF4F. This mechanism could also explain the reported effect of DUG homologues to inhibit tumour promoter-induced neoplastic transformation [28], since inhibition of translation could have a significant effect on neoplastic transformation.

To our initial surprise, DUG expression resulted in an inhibition of apoptosis induced by TRAIL, cycloheximide, and BADGE. This suggests that DUG may interfere with both the intrinsic and extrinsic pathways of apoptosis. Further investigations in our laboratory are underway to elucidate the mechanisms that are responsible for this DUG-mediated effect on apoptosis.

In summary, we have cloned and characterized a novel rat gene that is capable of regulating apoptosis. It is a homologue of eIF4G and is able to bind eIF4A, which may in turn be responsible for the rapid inhibition of protein synthesis observed in apoptotic cells.

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