Identification of proteins involved in transcription/translation (eEF 1A1) as an inhibitor of Bax induced apoptosis

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Abstract
Eukaryotic elongation factor 1A1 (eEF1A1) is central to translational activity. It is involved in complexes that form signal transduction with protein kinase C, as well as being a signal transducer and activator of transcription 3. eEF1A1 and eEF1A2 are isoforms of the alpha subunit of elongating factor 1 complex. It has been reported that eEF1A1 is expressed in most human tissues but the brain, skeletal muscle and heart. eEF1A1 has been linked to both apoptosis and anti-apoptotic activities. In this study, eEF1A1 was co-expressed with Bax, a proapoptotic protein via heterologous expression of recombinant DNA in yeast cells. Assays were carried out to monitor the fate and state of yeast cells when eEF1A1 was co-expressed with Bax. The yeast strain (bearing an integrated copy of the Bax gene) was transformed with an episomal 2-micron plasmid that encodes HA-tagged eEF1A1 gene. The resultant strain would allow co-expression of Bax and eEF1A1 in yeast cells, Bax being under the control of the GAL1 promoter, while the PGK1 promoter drives eEF1A1 expression. Bcl 2A1, a known anti-apoptotic protein, was also co-expressed with Bax in yeast cells as a positive control, to study the anti-apoptotic characteristic of eEF-1A1. The part eEF1A1 plays in apoptosis has been contentious, amidst the pro and anti-apoptotic properties of eEF1A1, it was shown clearly, in this study that eEF1A1 portrays only anti-apoptotic property in the presence of pro-apoptotic protein, Bax.

Keywords Human eEF-1A1 · Anti-Apoptosis · eEF-1A1 and yeast · Bcl-2A1 and yeast · Yeast apoptosis

Introduction
Eukaryotic elongation factor 1A proteins have multiple functions, which include aminoacyl-tRNAs recruitment during protein synthesis to ribosomes, actin building, phosphoinositol-4 kinase activation, and microtubule splitting [1]. Moreover, it was reported that human eEF1A1 might naturally form oligomers, and its overexpression has been linked to the transcription of muscle pro-apoptotic and proteolysis genes in hypercatabolic trauma patients [1]. eEF1A1 is vital for translation, and It is linked to the formation of signal transduction complexes with protein kinase C a well as being a signal transducer and activator of transcription 3 [2]. eEF1A1 and eEF1A2 originate from the alpha subunit of elongating factor 1 complex; there is about 90% similarity in the genes that encode the two isoforms [3]. eEF1A1 protein is widely expressed in different tissues while eEF1A2 is found mainly in the brain, skeletal muscle and heart [3]. According to Bosutti et al. eEF1A1 is expressed in all human tissues excepting the brain, skeletal muscle and heart. Its levels are gradually reduced at the primary phase of growth and development. It seems that eEF1A2 takes over the function of the synthesis of proteins from eEF1A1 in the adult muscle [4].

eEF1A1 has been described to be associated with apoptosis while eEF1A2 was linked to cell proliferation and inhibition of apoptosis in cultured myotubes [5]. The changes that occur in the expression levels of eEF1A1 and eEF1A2 are connected to anti-apoptotic and pro-apoptotic responses [5]. Sasikumar et al. stated that, in muscles, it seems that eEF1A1 acts as being a pro-apoptotic protein while eEF1A2 acts as being an anti-apoptotic protein.Remarkably, the two eEF1A isoforms act in opposite directions, during
differentiation. When eEF1A1 levels decrease, eEF1A2 levels increase [6]. Therefore, the switch between the two isoforms is important in muscle, brain, and heart, and it is critical for different stages of development of tissues [6].

The central and peripheral nervous system remyelination is required for active recovery after injury. Acetylated eEF1A1 has been implicated to adversely regulate the remyelination of the central and peripheral nervous system [7].

The eEF1A1 protein is expressed primarily in the cytosol of all tissues except the brain, skeletal muscle and heart [8, 9]. eEF1A1 protein has been found to influence transforming growth factor (TGF)-inhibited membrane-associated protein (TIMAP), a protein phosphatase 1, which is abundant in endothelial cells. Blanch et al. has reported that eEF1A1 interacts with p73 and p53 tumour suppressor proteins which are primary regulators of cell death and proliferation. Overexpression of eEF1A1 results in resistance to induction of apoptosis, mediated by p73 and p53, during chemotherapy. This may suggest that eEF1A1 subdues the pro-apoptotic activities of p73 and p53; hence, eEF1A1 may possess anti-apoptotic property. Moreover, high level of eEF1A1 has also been reported to protecting against stress-induced tumour cells death, which promotes cancer cell survival [10]. The p53 proteins play a massive role in tumorigenesis. eEF1As are transcription factors regulating many genes involved in cell cycle progression and apoptosis [3]. The eEF1A1 protein has been implicated in playing roles in the degradation of proteins, embryogenesis, organisation of the cytoskeleton, oncogenic changes, apoptosis, and proliferation of cells [3].

Overexpression of eEF1A1 has been described in tumours of the colon, pancreas, prostate, breast and lung, and melanomas [3]. It was suggested that eEF1A1 could be a prognostic marker and a therapeutic target for chronic lymphocytic leukaemia [11] and hepatocellular carcinoma [12, 13]. The two similar eEF1A isoforms are different in their participation in apoptosis [6]. There seems to be a correlation between the level of eEF1A and the percentage of cells undergoing apoptosis. There is a possibility that eEF1A proteins are involved in both pro-apoptotic and anti-apoptotic activities [3, 6]. The part eEF1A1 plays in apoptosis have been debatable. Blanch et al. [3] have indicated that the expression of eEF1A1 is amplified in response to oxidative stress, p53 activation and ER stress-induced apoptosis. Bcl 2A1 is an anti-apoptotic protein [14]. Human Bcl 2A1 gene has three exons and resides on chromosome 15q24.3 [15, 16]. Bcl-2 (B cell lymphoma 2) proteins are essential regulators of cell death and have a significant function in controlling cytochrome c release from the mitochondria during intrinsic apoptosis [14]. Bcl2A1 exercises primary pro-survival (anti-apoptotic) function. Eukaryotic elongation factors (eEF1A1 and eEF1A2) perform a crucial role in messenger RNA (mRNA) translation to protein. eEF1A2 is overexpressed in healthy humans skeletal muscle, while the expression of eEF1A1 in cellular stress models increased and linked to catabolism and apoptosis [1].

The role of eEF1A1 in apoptosis has not been evident, amongst the pro-apoptotic and anti-apoptotic characteristic of eEF1A1. eEF1A1 has been reported to interact with p73 and p53, which play a crucial role in the regulation of cell death [3]. Overexpression of eukaryotic translation elongation factor 1-alpha1 has also been linked with the inhibition of p73, p53 and apoptosis induced via chemotherapy (leading to chemoresistance) [3]. This might suggest that eEF1A1 regulate p53 and p73 pro-apoptotic function negatively. In the study, the anti-apoptotic feature of eEF1A1 was investigated by co-expressing eEF1A1 with a pro-apoptotic protein (Bax), via heterologous expression of recombinant protein. Assays were carried out to monitor cell death, ROS generation, mitochondrial membrane potential and nuclear DNA fragmentation (TUNEL assay).

Materials and methods

Yeast strains

The yeast strain W303-1A Mata (ATCC #208,352), is auxotrophic for the genes ADE2, HIS3, LEU2, TRP1 and URA3. New yeast strains were derived by transforming integrative plasmids (Supporting Information, Sect. 1, 2, and 3) which would express Bax from the GAL1 promoter or episomal plasmid expressing eEF 1A1 or Bcl 2A1 gene on PGK promoter.

Yeast transformation

Plasmids bearing Bax gene expression cassettes under the control of the galactose-inducible GAL1 promoter (GAL1p; see Supporting Information, Sect. 1) was used for genomic integration at the LEU2 chromosomal loci of the yeast strain to yield strains that contain 1 copy of Bax. And an episomal plasmid bearing eEF 1A1 or Bcl 2A1 gene expression cassettes on a PGK1 promoter. The transformation was carried out using a published protocol [17].

Detection of dead cells with phloxine B dye

Cell death was assessed by staining cells with the red dye Phloxine B (Sigma, P-4030-25 g) [18]. Live cells expel the dye, whereas it is accumulated in dead cells. This can be observed by fluorescence microscopy. Staining experiments were performed exactly as published earlier [19].
Detection of ROS

AAT Bioquest Fluorimetric Intracellular Total ROS Activity Assay Kit (#22,901) was used for measuring ROS. Experiments were performed as published earlier [19].

Quantifying mitochondrial membrane potential (MMP) with the JC-10 dye

AAT Bioquest JC-10 Mitochondrial Membrane Potential Assay kit (#22,800) uses water-soluble JC-10 to determine MMP quantitively. Experiments were conducted as per the published protocol [19].

Staining with hoechst dye for monitoring live cells

Hoechst 33,258 (Thermo Fisher Scientific; #H21491) is a nucleic acid stain widely used to detect live cells. When bound to double-stranded DNA, the dye emits blue fluorescence. Staining with the dye was performed as described earlier [19].

Assessing nuclear DNA fragmentation via the TUNEL assay: AA

AAT Bioquest TUNEL Apoptosis Assay kit (#22,844) was used for the detection of nuclear DNA fragmentation (NDF). The assays were performed as described earlier [19].

Western blotting

Western blotting was carried out using standard protocols [20], using primary antibodies specific to c-Myc (Thermo Scientific, #MA 1-980) and HA-tag (Proteintech, #51064-2-AP) or β-actin (Proteintech; #60008-1-Ig).

Results

Co-expression of human eEF 1A1 and Bcl 2A1 (from PGK1 promoter) with Bax from the GAL1 promoters in yeast

The yeast strain::Bax(LEU2) (with an integrated copy of the Bax gene at LEU2 locus) was transformed with an episomal 2-micron plasmid that encodes HA-tagged eEF1A1 gene. The resultant strain Bax(LEU2)::eEF1A1 would allow co-expression of Bax and eEF1A1 in yeast cells, Bax being under the control of the GAL1 promoter, while the PGK1 promoter drives eEF1A1 expression. Bcl 2A1 was also co-expressed with Bax from the strain Bax(LEU2):: Bcl2A1 as a positive control to study the anti-apoptotic characteristic of eEF-1A1.

Figure 1 showed the growth assays (on solid and liquid medium) for strains expression Bax gene with eEF1A1 (Bax(LEU2)::eEF1A1) (Fig. 1a, b) and with Bcl2A1 (Bax(LEU2)::Bcl2A1) (Fig. 1e, f). The death assay (Phloxin B assay) which shows the percentage cell death after co-expressing the genes along with their respective control was shown in Fig. 1c, d (for Bax(LEU2)::eEF1A1) and Fig. 1g, h (for Bax(LEU2)::Bcl2A1).

The mitochondrial potential was measured in the strains, as shown in Fig. 2a, d. Figure 2a showed the mitochondrial potential for strain expression Bax gene with eEF1A1 (Bax(LEU2)::eEF1A1) while Fig. 2d showed the mitochondrial potential for strain expression Bax gene with Bcl2A1 (Bax(LEU2)::Bcl2A1) along with respective control. Hoechst 33,342 dye stains live cells, the was shown in Fig. 2b, c for Bax(LEU2)::eEF1A1 and Bax(LEU2)::Bcl2A1 respectively.

Figure 3a, d shows the ROS measurement for Bax(LEU2)::eEF1A1 and Bax(LEU2)::Bcl2A1, respectively. Nuclear DNA fragmentation was measured using TUNNEL assay as shown in Fig. 3b, c for Bax(LEU2)::eEF1A1 and Fig. 3e, f for Bax(LEU2)::Bcl2A1 along with their controls. Figure 3g shows the western blots of the respective proteins. The western blot in Fig. 3g was used to monitor the presence proteins, panel (a) was probed with HA-tag antibody that recognizes HA-tagged eEF1A1 gene (lane 2) and HA-tagged Bcl-2A1 gene (lane 3), while lane 1 is the control. Panel (b) was probed with an antibody that recognizes the c-myc-tag Bax gene for the three above strains, while panel (c) was probed with actin antibody as a housekeeping protein.

Discussion

The results above show that the yeast strain on the galactose-containing (SG) plate that co-expressed eEF1A1 with Bax (Fig. 1a) grew while the strains which expressed Bax alone did not grow. This was similar for Bcl 2A1 (Fig. 1e, f), Fig. 1a, b show that eEF1A1 supports the growth of yeast cells carrying the toxic Bax gene (in solid and liquid media) when the expression of the pro-apoptotic protein Bax was fully induced in the presence of galactose. Apoptotic and anti-apoptotic proteins tend to neutralise each other when co-expressed. There are series of biochemical processes leading to the induction of apoptosis (e.g. mediated by pro-apoptotic proteins, Bak or Bax), on the one hand [35]. Cell survival (e.g. mediated by anti-apoptotic proteins, Bcl-2 or Bcl-xL) compete to regulate mitochondrial permeability cells as this is one of the main targets of proapoptotic protein, where increasing the ROS generation [21]. One of the events that take place during the induction of apoptosis includes, drafting of Bax to the outer membrane of the mitochondria along with Bak. Thereby permeabilising the
Comparing the growth of yeast strains Bax(LEU2)::eEF1A1 and Bax(LEU2)::– in minimal liquid SG medium that contains galactose

Fig. 1  a, e Growth of yeast strains in solid agar plates over 72 h, in glucose-containing minimal medium (SD), and galactose-containing minimal medium (SG). The 4 strains on the upper half of the two plates are Bax(LEU2):: eEF 1A1 (a) and Bax(LEU2)::Bcl-2A1 (e) transformants (1), containing an episomal 2 µ-plasmid plasmid that encodes the HA-tagged eEF 1A1 (a) and Bcl-2A1 (e) genes and a Bax expression cassette integrated at the LEU2 locus. The 4 strains, in the lower half of the plates (a, e), are Bax(LEU2)::– (2) transformants which contain the Bax expression cassette and an empty plasmid. b, f Growth of yeast cells, Bax:: eEF 1A1 (b) and Bax::Bcl-2A1 (f), in minimal liquid medium containing galactose, throughout 48 h along with control strain. A two-tailed paired sample t-test show statistically, that there was a significant difference (p < 0.05). c, g The percentage cell death in strains Bax:: eEF 1A1 (c), Bax::Bcl-2A1 (g) and control Bax(LEU2)::–, after growth in galactose for 48 h. Dead cells stained with phloxine B are shown in (d, h). A two-tailed paired sample t-test show statistically, that there was a significant difference (p < 0.05)
membrane by forming pores on the membrane [22]. Bcl 2A1 is a family of Bcl-2 protein [14]. Bcl-2 (B cell lymphoma 2) proteins are essential regulators of cell death. They have a significant function in controlling cytochrome c release from the mitochondria during intrinsic apoptosis [14]. Bcl 2A1 exercises essential pro-survival function.

Phloxine B and Hoechst staining demonstrated the extent of death and life present in the cells, as shown in Figs. 1c, d, g, h and 2b, c. Huang et al. [23] reported that after knocking down eEF1A1 gene in human acute T lymphocytic leukaemia cell line Jurkat, there were visible effects on the induction of apoptosis and inhibition of proliferation of Jurkat cells, which could be due to the knockdown. Enhancement of cell proliferation was observed in eEF1A1 expressing group along with inhibition of apoptosis [24]. eEF 1A1 may have an essential role in the progression of cancerous or tumour cells. The results of this study show that eEF1A1 appear to be anti-apoptotic when co-expressed with Bax (a pro-apoptotic protein). It was observed that, in yeast cells, much less cell death occurs in cells that co-express eEF1A1 than cells which express Bax alone, indicating that eEF1A1 protects yeast cells from Bax-mediated cell death, a similar result was obtained with Bcl 2A1. The results show that eEF1A1 and Bcl 2A1 protects the mitochondria of cells co-expressing Bax from loss of membrane potential (Fig. 2a, d). A crucial source of ROS generation is the mitochondria [25–27], the release of electron-induced by Bax results in the production of ROS, which could trigger apoptosis [21]. Damage to mitochondria can result in a surge of ROS generation and further destruction of the components of the mitochondria, leading to more ROS production and further damage [26]. Measurement of ROS produced in yeast cells Bax(LEU2):: eEF 1A1 (Fig. 3a), Bax(LEU2):: Bcl2A1 (Fig. 3d) cells, and Bax(LEU2)::—cells expressing Bax alone show that the difference in ROS production is significant between the strains. Cells often produce some metabolites with antioxidant action in response to ROS species, which could be lipophilic or hydrophilic, and cells can also provide a variety of enzymes with antioxidant activity [28]. Induction of these processes usually leads to repair, detoxification, or the maintenance of homeostasis of metal ions. Blanch et al. has reported that eEF1A1 interacts with p73 and p53 tumour suppressor proteins which are central regulators of cell death and proliferation. Hence, eEF1A1 may possess anti-apoptotic property. They are transcription factors regulating many genes involved in cell cycle progression and apoptosis [3]. It also has other functions and has been referred to as a moonlighting protein (i.e. a protein having multiple roles) [8]. eEF1A1 has reported interacting with proteins that contain Polyalanine or poly(A), suggesting that eEF1A1 controls the expanded poly(A) proteins subcellular locality and could prospectively be a target for therapeutic for tackling of poly(A) diseases pathogenesis.

![Fig. 2](image)
**Fig. 3**  
(a, d) Measurement of ROS produced in the yeast strains Bax(LEU2)::eEF1A1 (a), Bax(LEU2)::Bcl-2A1 (d) and Bax(LEU2)::−, a two-tailed paired sample t test show that there was significant difference ($p < 0.05$).  
(b, e) TUNEL assay comparing the nuclear DNA fragmentations in yeast strains Bax(LEU2)::eEF 1A1 (b), Bax(LEU2)::Bcl 2A1 (e) and Bax(LEU2)::−. The cells Bax(LEU2)::eEF 1A1 and Bax(LEU2)::Bcl 2A1 have lower nuclear DNA fragmentation compared to Bax(LEU2)::− cells. A two-tailed paired sample t test show that there was significant difference ($p < 0.05$).  
(c, f) represent microscopic image of cells after tunnel assay, Bax(LEU2):: eEF 1A1 (c) and Bax(LEU2)::Bcl 2A1 (f).  
g Western blot to monitor the presence of Bax in yeast cells that express only Bax and in cells that co-express Bax together with eEF-1A1 and, Bcl-2A1. 10 µg of total cellular proteins, in cell lysates, were loaded on to each lane and the blot was probed with an antibody that recognizes the c-myc tag, Actin, & HA-tag. The plasmid pRS305/GAL1p-h_bax-MS has restriction sites that cut the plasmid only once (Supporting information Fig. 1). The human Bax gene contains, at the 3′-end, a sequence that codes for the c-myc tag so that expressed protein can be monitored with an antibody that recognizes the c-myc epitope at the C-terminus of Bax protein. Plasmid pSYE239/eEF1A1-HA (Supporting information Fig. 2) is an episomal 2µ-plasmid encoding HA-tagged eEF1A1 gene. The plasmid pSYE239/Bcl-2A1-HA is an episomal 2µ-plasmid encoding HA-tagged Bcl-2A1 gene. (Supporting information Fig. 3)
It has been speculated that changes in expression levels of these two elongation factors may relate to their pro and anti-apoptotic responses [5, 30]. Western blotting was performed to confirm the expression of the respective protein, as shown in Fig. 3g.

Bcl2A1 is a known anti-apoptotic protein, with the similarity in the result obtained compared to that of Bcl2A1, this suggests that eEF1A1 possess anti-apoptotic property in the presence of a proapoptotic protein (Bax). It has been reported that the part eEF1A1 plays in apoptosis have been unclear. However, eEF1A1 has been found to interact with Cytotoxin-associated gene A protein in gastric adenocarcinoma, whereby increasing STAT3S727 phosphorylation in the nucleus [31, 32]. eEF1A1 expression could be an objective prognostic factor linked with death and also for disease survival, elevated eEF1A1 expression has a favourable prognostic impact on colon adenocarcinoma patients [32]. eEF1A1 may also serve as a valuable prognostic biomarker and promising therapeutic target of renal cell carcinoma [33]. The above results clearly show that, in the presence of Bax, eEF1A1 was anti-apoptotic. Hence, eEF1A1 possesses an anti-apoptotic characteristic, which may have implications in the progression of some diseases.

Conclusions

eEF1A1 sharp decrease in excitatory synapses was observed in mouse model pathology, at the initial stages of human alpha-synuclein, eEF1A1 might mean a reducing factor to the maintenance of synapses [34, 35]. Bax is one of the primary proteins deployed during apoptosis, eEF1A1’s role in the translation of proteins could directly or indirectly be linked to its anti-apoptotic function exhibited in this research. In summary, considering the above observation, we can conclude that eEF 1A1 was able to rescue Bax induced apoptosis and that it is anti-apoptotic. Amidst the pro and anti-apoptotic role of eEF1A1, it has been shown clearly, in this study that eEF1A1 portrays only anti-apoptotic property in the presence of the proapoptotic protein, Bax. More interestingly, this could have an impact in the pathogenesis, treatment and prognosis of the diseases including cancer and neurodegenerative diseases.

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Author contributions DA performed all the experiments. BC coordinated the study. BC and DA wrote the manuscript.

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Compliance with ethical standards

Conflicts of interest DA declares that he has no conflict of interest. BC declares that he has no conflict of interest.

Research involving human participants and/or animals No.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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