

Bacterial toxin RelE induces apoptosis in human cells

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Abstract The bacterial protein RelE severely restricts prokaryotic cell growth, probably by acting as a global inhibitor of translation. It is ubiquitous in prokaryotes as part of the RelE–RelB toxin–antitoxin system, and may be activated by nutritional stress. When the *relE* gene from *Escherichia coli* was expressed inducibly in a human osteosarcoma cell line, it was shown to retard growth and to lead to cell death by apoptosis. RelE is therefore unusual among bacterial toxins in possessing broad activity against both prokaryotes and eukaryotes, perhaps by acting on evolutionarily conserved components of the translation machinery. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: RelE; RelB; Toxin–antitoxin; Mammalian cell; Human cell; Growth inhibition

1. Introduction

The *Escherichia coli* K-12 *relBEF* operon encodes three proteins: RelE and RelB comprise a toxin–antitoxin module [1,2], while RelF is a member of the Hok cytotoxin family [3]. RelE is an 11.2 kDa basic protein and is cytotoxic or cytostatic when overexpressed in bacteria [2] due to a marked inhibition of translation [4], which is probably mediated by a direct interaction with ribosomes [5]. RelB is a 9.1 kDa acidic protein which forms a strong complex with, and acts to neutralise, RelE; it is relatively unstable, being susceptible to Lon protease activity, and is also an autorepressor of the *relBEF* operon [2,4]. The precise function of RelE–RelB is unclear: when located on plasmids it may act as a stabilisation system by post-segregational killing [2,6], but the presence of *relBE* genes on the chromosomes of many bacterial species, both eubacterial and archaeal [7], is inconsistent with this. More recently, the RelE–RelB module has been proposed to function as a stress response element, being activated by amino acid starvation and serving to downregulate translational activity within the cell [4].

Prokaryotic protein toxins which act on bacteria are not normally known to affect eukaryotic cell types, but some evidence suggests that RelE might also inhibit growth of the yeast *Saccharomyces cerevisiae* [8]. This is a surprising finding since only one other protein of bacterial origin, Shiga toxin (STX) and its relatives from *Shigella dysenteriae*, exerts toxic

effects on both prokaryotic and eukaryotic cells [9]. To test whether RelE is toxic in higher eukaryotes, the *relE* gene from *E. coli* K-12 has been introduced under control of an inducible promoter into the human osteosarcoma cell line U2OS. It is shown that expression of the *relE* gene in human cells leads to growth inhibition and cell death by apoptosis.

2. Materials and methods

2.1. Gene expression constructs

The *E. coli* K-12 *relE* gene was amplified by polymerase chain reaction (PCR) from plasmid pBD2430 [10], using the oligonucleotide primers 'relE-5'' (5'-GTCGACCATGGCGTATTTCTGGATT-TG-3') and 'relE-3'' (5'-GGTACCTCAGAGAAATGCGTTTGACCG-3'), and its sequence confirmed after cloning in pCR2.1-TOPO (Invitrogen, Groningen, The Netherlands) as pCR2.1-TOPO-*relE*. The *relE* gene was excised as a *Pst*I–*Bam*HI (blunt-ended) fragment and ligated in the sense orientation into expression vector pcDNA4/TO (Invitrogen), digested with *Pst*I–*Not*I (blunt-ended), giving plasmid pcDNA4/TO/*relE*. This was then modified by replacement of the zeocin resistance gene with a cassette providing G418 resistance in mammalian cells: a *Spe*I–*Not*I fragment from pCEP9β (a gift of Mark Tykocinski, University of Pennsylvania School of Medicine) was made blunt-ended using Klenow fragment, and this was ligated to pcDNA4/TO/*relE* digested with *Pvu*II, giving plasmid pcDNA4/TO/*relE*(G418^R). The *relE* gene was also inserted into pcDNA4/TO in the antisense orientation from pCR2.1-TOPO-*relE* as a *Bam*HI–*Not*I fragment to give pcDNA4/TO/*Eler*. Constructs were confirmed by restriction analysis and sequencing.

2.2. Cell lines and transfection

The human osteosarcoma TReX-U2OS cell line constitutively expressing tetracycline repressor from pcDNA6/TR (Invitrogen) was routinely grown at 37°C and 5% CO₂ in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum, 10 mM glutamine, 500 U/ml penicillin and 0.5 mg/ml streptomycin (Sigma-Aldrich, Poole, UK). U2OS cells were transfected using GeneJammer reagent (Stratagene, Amsterdam, The Netherlands) following manufacturer's instructions. Briefly, an 80% confluent 35 mm dish with 900 µl fresh serum containing DMEM was incubated for 3 h at 37°C and 5% CO₂ in the presence of 100 µl DMEM, 6 µl GeneJammer reagent and 1 µg DNA mixture after which an equal volume of serum-containing DMEM was added. Cells were incubated at 37°C and 5% CO₂ for an additional 24 h then split 1:5 into growth medium. After a further 24 h transfectants were selected in growth medium supplemented with 1 mg/ml G418 (Geneticin, Sigma-Aldrich) or 250 µg/ml zeocin (Invitrogen), depending on the construct used. Multiple transfections were done in six-well plates for each construct, selecting only one clone from each well. Transfectant clones generated with pcDNA4/TO/*relE*(G418^R) were designated U-*relE*; those containing pcDNA4/TO/*Eler* were designated U-*Eler*.

2.3. Growth curve

U2OS transfectant clones were seeded at $\sim 5 \times 10^4$ cells each in T25 flasks. After 12 h incubation half of the flasks were induced with 5 µg/ml tetracycline and an equal volume of absolute ethanol added to the other flasks as a control. Every 12 h for 72 h, starting from 0 h after

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induction, one induced flask and one control flask were washed with phosphate-buffered saline (PBS; pH 7.4), trypsinised and counted with a haemocytometer (at least six counts per sample). Medium, including tetracycline where appropriate, was replaced every 24 h in flasks not being harvested.

2.4. MTT assay

Between 1×10^4 and 5×10^4 cells were seeded in each well of a six-well (35 mm diameter) plate. After 12 h incubation at 37°C wells were treated with 5 µg/ml tetracycline or absolute ethanol as a control. Every 12 h for 72 h, starting from 0 h after induction, one induced well and one control well were incubated with 5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich) for 6 h at 37°C, then centrifuged at 2000 rpm for 10 min. The medium was then aspirated off, 1 ml DMSO added, mechanically mixed until crystals were dissolved and absorbance read in a micro-culture plate reader at a test wavelength of 550 nm and a reference of 660 nm.

2.5. RT-PCR

For each clone, approximately 10^7 cells were harvested. The Absolute RNA Miniprep Kit (Stratagene) was used to isolate total RNA; mRNA was purified using PolyATtract Systems III and IV (Promega, Southampton, UK), both according to manufacturer's protocol. RT-PCR was performed using the Access RT-PCR Kit (Promega) according to manufacturer's instructions using the *relE*-5' and *relE*-3' primers. Samples were taken from each PCR after 10, 20 and 30 cycles.

2.6. Cloning efficiency

U-*relE* and U-Eler clones were plated at various densities (up to 10^7 cells per 15 cm dish) either with or without tetracycline induction 12 h after seeding. After incubation at 37°C for a further 10 days, medium was removed, plates were washed twice with PBS, stained with Leichmann's staining solution (eosin methylene blue; BDH Merck, Poole, UK) for 3 h, rinsed gently in warm water and air-dried. Colonies counted were related to number of cells seeded to calculate cloning efficiency.

2.7. DNA fragmentation analysis

Approximately 5×10^6 cells each were induced with 5 µg/ml tetracycline, an equal volume of absolute ethanol or 1 µg/ml actinomycin D (Sigma-Aldrich), 12 h after seeding. Floating cells were collected every 12 h for 72 h, replacing fresh pre-warmed medium and the appropriate amount of tetracycline, absolute ethanol or actinomycin D each time, and stored at -20°C until processing. The Suicide Track DNA Ladder Isolation Kit (Oncogene Research Products/CN Biosciences UK, Nottingham, UK) was used to isolate total DNA and visualise samples according to manufacturer's protocol.

3. Results

3.1. Growth curve and metabolic activity

To examine the effect of *RelE* in human cells, the osteosarcoma cell line U2OS was transfected with constructs containing the *relE* gene in the sense orientation, or, as a control, in the antisense orientation, giving cell lines designated U-*relE* or U-Eler, respectively. Gene expression was controlled by Tet operator sequences in the promoter region, to which the constitutively expressed Tet repressor protein binds; expression was induced by addition of tetracycline. A typical growth experiment for one U-*relE* clone and one U-Eler clone, with or without tetracycline, is shown in Fig. 1.

The number of tetracycline-induced U-*relE* cells that adhere to the flask steadily decreased after 12 h, suggesting that *relE* expression was detrimental to cell growth. In contrast, uninduced U-*relE*, and both induced and uninduced U-Eler cells, maintained steady growth. Expression of the antisense *relE* gene apparently has no effect on growth. However, uninduced U-*relE* cells grew at a slower rate than U-Eler cells, which was attributed to a leaky promoter, and/or trace amounts of tetra-

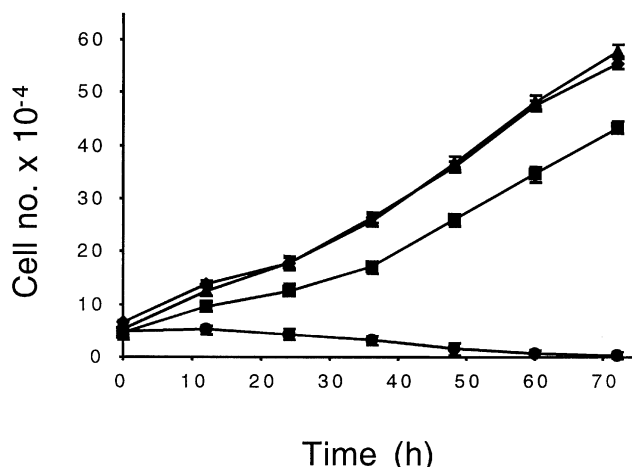


Fig. 1. Growth curve of U-*relE* and U-Eler with and without induction of sense and antisense *relE*. U-*relE* cells [(induced: filled circle) and (uninduced: filled square)] were harvested every 12 h for 72 h after induction and counted. U-Eler cells [(induced: filled diamond) and (uninduced: filled triangle)] were processed in parallel. Vertical bars indicate standard deviation.

racycline present in the growth medium, giving rise to low level expression of *relE*. Similar growth curves were obtained when the experiment was repeated, and when other, independent U-*relE* and U-Eler clones were used.

The effect of *relE* expression on the metabolic status of the cell was tested using the MTT assay, which measures activity of mitochondrial dehydrogenases. A pattern similar to that obtained from the growth curves was revealed (Fig. 2): metabolic activity of tetracycline-treated U-*relE* cells declined after 12 h, whereas activity of uninduced U-*relE* cells, and both induced and uninduced U-Eler cells, increased steadily, reflecting cell growth. The effect of *relE* expression on the metabolism of mammalian osteosarcoma cells is clearly inhibitory.

3.2. Transcription of *relE* sense and antisense genes

PCR analysis confirmed that the *relE* sense and antisense constructs were intact in genomic DNA of their respective transfectant cell clones (data not shown). Gene transcription

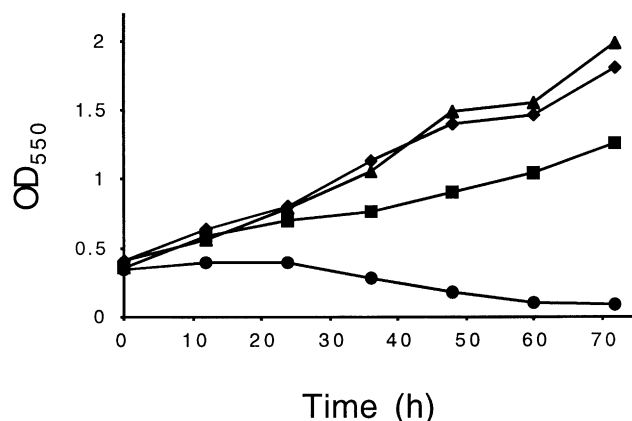


Fig. 2. Metabolic activity in U-*relE* and U-Eler clones with and without induction of sense and antisense *relE*. The MTT assay was performed on tetracycline-treated (filled circles) and control U-*relE* cells (filled squares) starting from 0 h after induction. Treated (filled diamonds) and control U-Eler cells (filled triangles) were assayed in parallel.

was established by RT-PCR, with samples taken after 10, 20 and 30 cycles of PCR (Fig. 3). Tetracycline-induced U-relE cells yielded a ~ 350 bp RT-PCR product from 10 cycles of the PCR, indicating *relE* transcription. In uninduced U-relE, the same product was seen, but not until 30 cycles of PCR, confirming the suggestion from Figs. 1 and 2 that the promoter is leaky, giving basal level transcription even in the absence of inducer. U-Eler cells showed a similar pattern of expression of the antisense gene under control of the same promoter and confirmed that antisense *relE* expression is not detrimental.

3.3. *RelE* killing efficiency

U-relE and U-Eler clones were plated at various densities either with or without tetracycline induction. U-relE cells failed to form colonies when *relE* expression was induced, whereas uninduced U-relE and induced/uninduced U-Eler cells showed approximately 50% plating efficiency (data not shown). The total number of cells plated was 2×10^8 , suggesting that, assuming 50% plating efficiency without *relE* expression, less than 1 in 10^8 cells survive expression of the toxin gene on induction. Intriguingly, the relatively low level of *relE* expression observed in uninduced U-relE cells, which apparently affects growth, had little effect on cloning efficiency in this experiment.

3.4. DNA fragmentation

When tetracycline-induced U-relE cells were visualised under a light microscope, morphological changes including membrane budding, chromatin condensation and fragmentation and reduction in cell volume were observed, all of which are characteristic of apoptotic cell death [11]. Uninduced U-relE and induced/uninduced U-Eler cells exhibited the normal

U-relE U-Eler

M +T +A C +T +A C

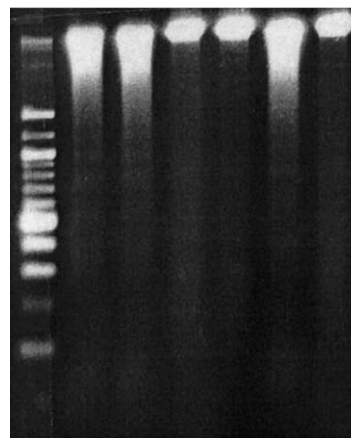


Fig. 4. DNA fragmentation assay showing genomic DNA from U-relE and U-Eler cells treated with tetracycline (+T), actinomycin D (+A) or untreated (C). M is a 100 bp ladder.

morphology of U2OS (results not shown). A typical ladder pattern of DNA degradation, due to a caspase-activated DNase, is also associated with apoptosis [12]. This was observed in tetracycline-induced U-relE cells and was comparable to apoptotic fragmentation induced by actinomycin D; the effect was not seen in uninduced cells. No fragmentation was observed in tetracycline-treated or control U-Eler cells, although actinomycin D was able to induce DNA degradation (Fig. 4).

4. Discussion

When expressed in a human cell line, the *E. coli* K-12 *relE* gene retards growth and results in cell death by apoptosis. In bacteria, RelE acts as a global inhibitor of translation [4], probably due to its direct interaction with ribosomes [5]. RelE might function in a similar manner in eukaryotic cells and indeed a number of ribosome-inactivating proteins (RIPs) are known which exhibit activity in both prokaryotic and eukaryotic cells. However, RIPs, the majority of which are derived from plants, are generally considerably more effective against eukaryotic ribosomes than their prokaryotic counterparts, typically by *N*-glycosidase activity on a conserved sequence in the 28S rRNA of the large subunit [13]. STX and its relatives, from *S. dysenteriae* and certain strains of *E. coli*, are the only bacterial RIPs which inhibit both prokaryotic and eukaryotic translation machinery, apparently with equal activity against both [9,14]. There are significant differences between STX and RelE; however, for example, STX is a secreted type II RIP, composed of an A (active) chain, and a B subunit which facilitates entry into the target cell, whereas RelE forms part of an intracellular toxin–antitoxin module with RelB. STX A chain acts catalytically to irreversibly inactivate ribosomes, whereas RelE action seems to be reversible if RelB is present [15]. The mode of action of RelE seems likely to be unusual or unique, therefore, and deserves further investigation.

One common feature of RelE and RIP action is the induc-

U-relE U-Eler

– Tet

+ Tet

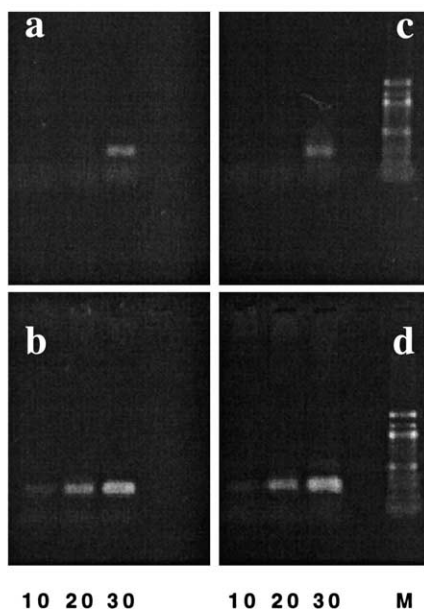


Fig. 3. Agarose gel of RT-PCR samples after 10, 20 and 30 cycles. Template mRNA from the following cells was used: untreated (a) and tetracycline-treated (b) U-relE cells; untreated (c) and tetracycline-treated (d) U-Eler cells. M is a 100 bp ladder.

tion of apoptosis. This endogenous cellular process operates via at least two pathways, and is characterised by chromatin condensation and degradation, membrane budding, cell volume reduction and nuclear matrix solubilisation leading to cell death [11]. RIPS are known to induce mammalian cell death by apoptosis [16–20]. The mechanism which triggers apoptosis is currently unclear, but is apparently downstream of 28S rRNA cleavage and inhibition of protein synthesis in the case of α -sarcin. Inhibition of protein synthesis per se using cycloheximide does not induce apoptosis, however [20]. Although RelE might inhibit translation in mammalian cells, it is unlikely to be a RIP (see above), suggesting that investigation of its effect on eukaryotic ribosomes will provide further insight into triggering of apoptotic pathways.

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