RNGB: a *Dictyostelium* RING finger protein that is specifically located in maturing spore cells

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Abstract The RING finger is a form of zinc finger motif found in proteins of widely varying biological function. The *Dictyostelium* RNGB protein contains a RING finger and also a K-box, a sequence motif found in several plant homeotic proteins. The *rngB* mRNA is present at low concentration in growing cells and gradually increases in abundance throughout development. However, the RNGB protein is not detected until culmination and we present evidence that suggests it is translationally regulated. The protein is specifically localised in maturing spore cells and is cytoplasmic, suggesting that the RING finger does not function as a DNA binding domain.

Key words: RING finger protein; *Dictyostelium discoideum*; *rngB* gene

1. Introduction

RING finger domains are present in many different eukaryotic proteins [1]. The family members are termed RING finger proteins because this motif is found in the human RING1 gene [2]. The RING finger contains seven cysteine and one histidine residues and these residues span a much larger finger region than is present in the zinc fingers of the steroid receptors and other transcription factors [1]. In the case of the RING1 protein zinc has been shown to be bound within the RING finger [3]. Some of the proteins that contain a RING finger motif are known to be, or are believed to be, transcription factors [4,5]. However, there are members of the family that clearly serve some quite different function, because they are found in the peroxisomes or in the vacuoles [6,7].

Dictyostelium is a valuable system in which to study gene function, because it is haploid and readily amenable to genetic analysis. Also, analysis of the developmental cycle sometimes reveals phenotypic effects of mutations that may be cryptic within vegetatively growing cells [8]. As part of a search for a regulator of *Dictyostelium* gene transcription, we serendipitously cloned a novel member of the RING finger family. We present its characterisation and show that it falls into the class of RING finger proteins that is extra-nuclear.

2. Materials and methods

2.1. Cell culture and development

Dictyostelium discoideum: AX-2 [9] cells were cultured and transformed as described previously [10,11] and the transformant clones were selected and grown in the presence of 20 μg/ml G418. For development, harvested cells were washed three times in KK_2 (16.5 mM KH_2PO_4, 3.8 mM K_2HPO_4, pH 6.2) and spread onto 0.45 μm nitrocellulose filters (Millipore) or onto 2% non-nutrient Bacto agar (Difco) plates.

2.2. Construction of the cDNA clone

Polyadenylated RNA from first finger stage *D. discoideum* cells was prepared by guanidium-HCl lysis and purified using PolyAtract 1000 (Promega). The first strand of the cDNA was synthesised by both random hexamer and by oligo(dT) priming, using 10 μg of polyadenylated RNA in each case, and equal amounts of cDNA synthesised with the two different methods were combined together. Following the second strand synthesis, the cDNA was rendered blunt-ended using T4 DNA polymerase and then *Bst*XI adapters were added [12]. Unligated adaptors were removed by passing through a Sephacryl S3000 (Pharmacia) spin column. The cDNA was size fractionated using a Sephacryl S400 (Pharmacia) spin column, which removed cDNAs shorter than 250 bp. Fractionated cDNA was then ligated into gel purified, *Bst*XI digested pSD.10 vector [13].

2.3. Northern blot analysis

For the preparation of RNA 2×10^7 AX-2 cells were allowed to develop on 0.45 μm nitrocellulose filters (Millipore) as described above. Poly(A)$^+$ RNA was isolated from different stages of development using PolyAtract 1000 (Promega). For the samples isolated from 20 and 24 h of development, cells were ground in liquid nitrogen before being purified. 1.5 μg of poly(A)$^+$ RNA was fractionated on a 1.2% agarose gel, containing formaldehyde, and blotted onto a Hybond-N$^+$ filter. The pre-hybridization and hybridization were carried out at 37°C in 50% formamide, 6×SSC, 10×Denhardt's solution, 1% SDS, and 100 μg/ml sonicated, boiled salmon sperm DNA for 2 and 16 h, respectively. The final hybridisation solution contained ^{32}P-labelled *rngB* probe made using a random prime labelling kit (Boehringer). PCR amplified DNA fragments were used as probes, in order to exclude repeated sequences in the cDNA. The hybridised membranes were washed three times in 2×SSC, 0.1% SDS at 65°C for 20 min and exposed to X-ray film (Kodak X-Omat) at −70°C for 1 week.

2.4. Preparation of the monoclonal antibody

A peptide (K2) corresponding to the carboxy-terminal hydrophilic region, LKQIGSIKDQ (residues 866–875 of the deduced RNGB sequence), was synthesised on a model 430A Applied Biosystems solid-phase synthesiser. The peptide was conjugated to two different carrier proteins, keyhole limpet haemocyanin (KLH, Sigma) and bovine serum albumin (BSA, Sigma). The peptide-protein conjugates (K2-KLH and K2-BSA) were subjected to gel filtration to remove free peptide. Mice were immunised with 1 mg K2-KLH peptide conjugate, emulsified with incomplete Freund's adjuvant, at 4-week intervals. Hybridomas were prepared and positive cells were screened by ELISA against K2-BSA and tested by Western blotting on RNGB protein expressed in bacteria. The positive cultures were cloned by dilution and then cultured for 1 week and tested by ELISA as above.

2.5. Western blot analysis and immunostaining with anti-RNGB peptide antibody

For the preparation of protein samples, 2×10^7 AX-2 cells were allowed to develop on 0.45 mM nitrocellulose filters (Millipore) as above. Total proteins were extracted from various developmental stages and from stalk or spore cells by suspending the harvested cells in SDS-sample buffer, boiling and then sonicating to break down the genomic DNA. Approx. 40–50 μg of the proteins were loaded onto either 7.5% or 10% SDS polyacrylamide gels and blotted onto a Hybond-C filter. The filter was incubated with anti-RNGB antibody at

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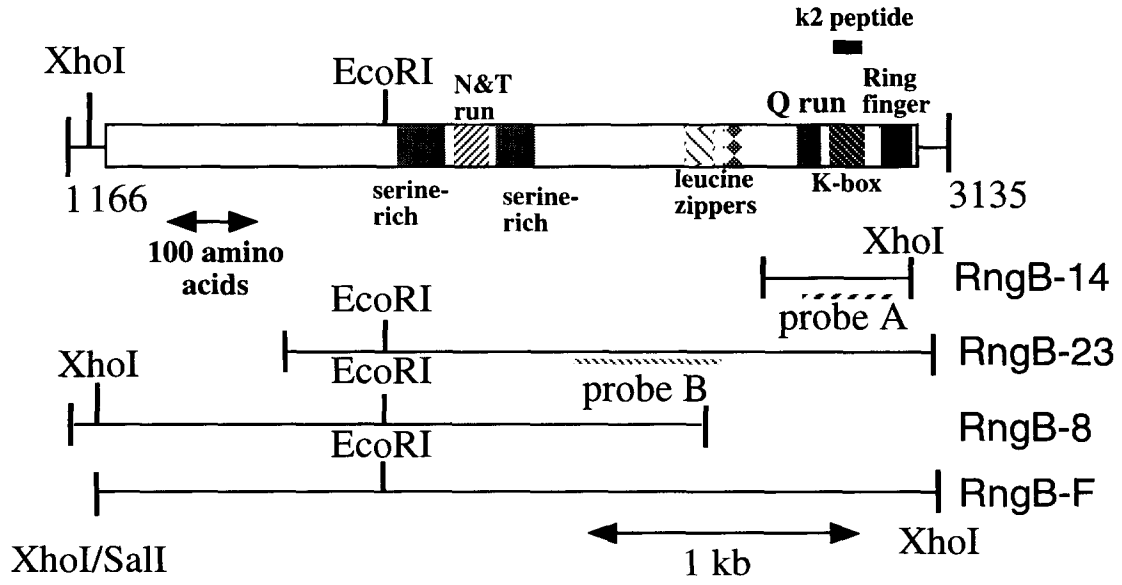
A.



B.

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MKIDCKRVSL IGSPEPRWGH TGTTLPNGSG FIVFGGNSNR  40
AFNDIQYYNI FNNSWSKIEA VGNAPSERYG HSAVLYQSQS  80
RPYSDSYQII FFGGRATSKP FSDINILYVN SNRSFIWKQV 120
TTKSIEGRAG HTAVVYRQNL VVFGGHNNHK SKYYNSVLLF 160
SLESNEWRQQ VCGGVIPSAR ATHSTFQVNN NKMFIFGGYD 200
GKKYYNDIYY LDLETWIWKK VEAKGTPPKP RSGHSATMIQ 240
NNKLMIFGGC GSSSNFLNDI HILHIEGANE YRWEQPSYLG 280
LEIPQARFRH TTNFIGGRVY IYAGTGSGNL MGDLHTLEFL 320
DDNNTPLIPI TISIPITNSN SIVGSPNTSI SCGVSNSGAS 360
SSSGGGISGH PSILSSSSSS SYLSTSPLST SSLASSYQSS 400
QSLQFNQNQN QNNNNNNNNN NNNNIQTTTT TTTNNNNNNN 440
NNNNNNNNNN NVESNQQQQQ IQHQTSPMSV LSRSNSNISL 480
NSLNSSSSSI LSTPSTLSTT TTTTTTSHAS HTSHTSNRSN 520
GSRGGIPSIP PFNGRSSNHN NNNNSNSNNY NNHQQTKTNS 560
AEELILEELK SLNIYDQAAC NKDFQTNLKR VEELFNQKIK 600
HEQKYRQSLE EKLGKANHQV SLLTNQIQSI IQKDELTSLK 640
KEYSELKKKH SLLYSEDIDD LPTETCLKLE EIHVKSLEKL 680
RVKKLPSSNQ LSTLQQQIPQ QPTTIICNNS QIIQQQPLPP 720
LQQQQQQQQQ QQQQQQQQQQ PLEIQEQLTM LQLQLSQLSQ 760
QQQNQIDKQQ KQEKLQQEQQ QQQLKNINRL SISSNSSTLS 800
SKDSFYFESK IQELSNQLKE KQQAITDRDN KIKDFENQLN 840
KYKLIGLDSM DHYQLLELES SFHNGLKQIG SIKDQRYLNR 880
LVSLEKEKDQ LKDQNSCVIC ASNPPNIVLL PCRHSSLCSD 920
CCSKLTKCPI CRSHIENKIS IYQ                   943
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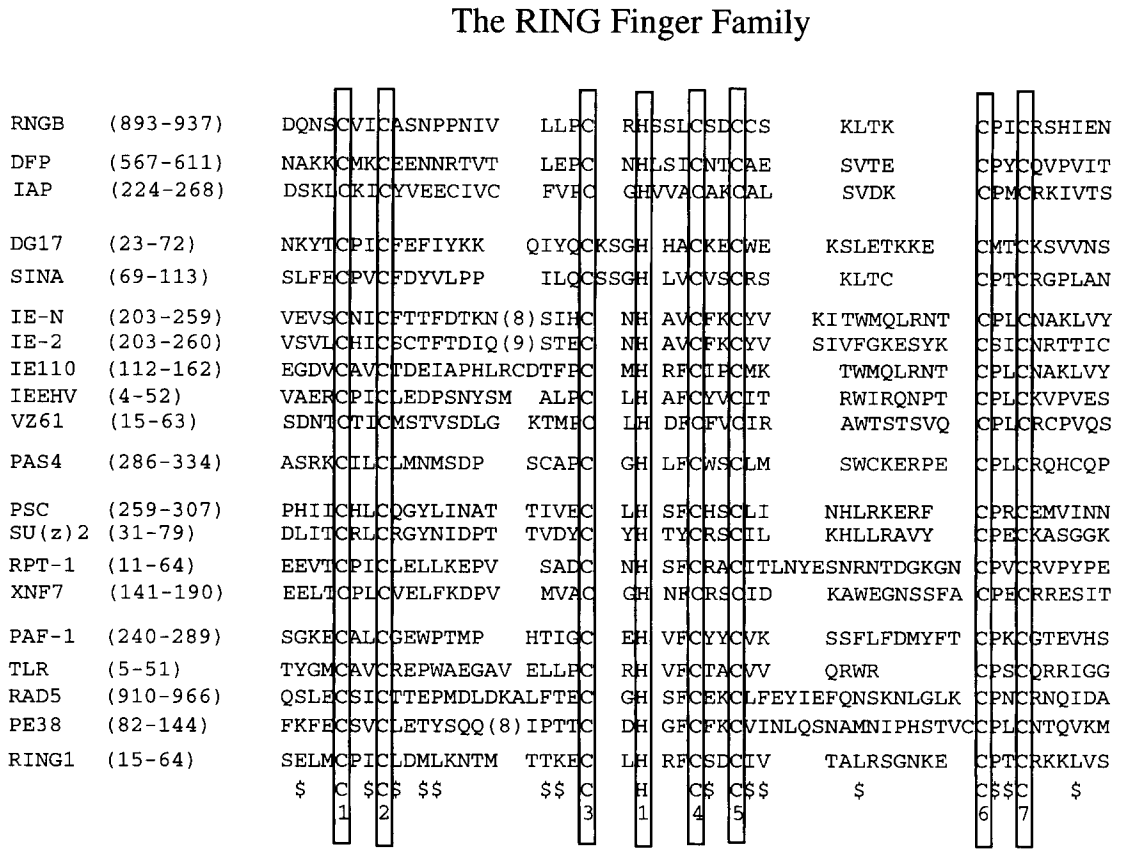
Fig. 1. Organisation of the *rngB* gene and deduced amino acid sequence of RNGB protein. (A) Scheme of the structural features of *rngB*. rngB-14 was initially isolated by genetic screening in yeast and the other clones were isolated by screening the cDNA library with probes A or B. RngB-F, which contains the entire open reading frame, was constructed as described in Section 2. The position of the peptide (K2) used for immunisation is shown by a solid bar. (B) Deduced amino acid sequence of the RNGB protein. The homopolymeric runs of asparagine and glutamine, the serine-rich domains, the basic region, the K-box homologous region, and the Ring finger domains are underlined. Conserved amino acids in the K-box homology region, Ring-finger domains and leucine repeats are shown in bold letters.

A.



B.

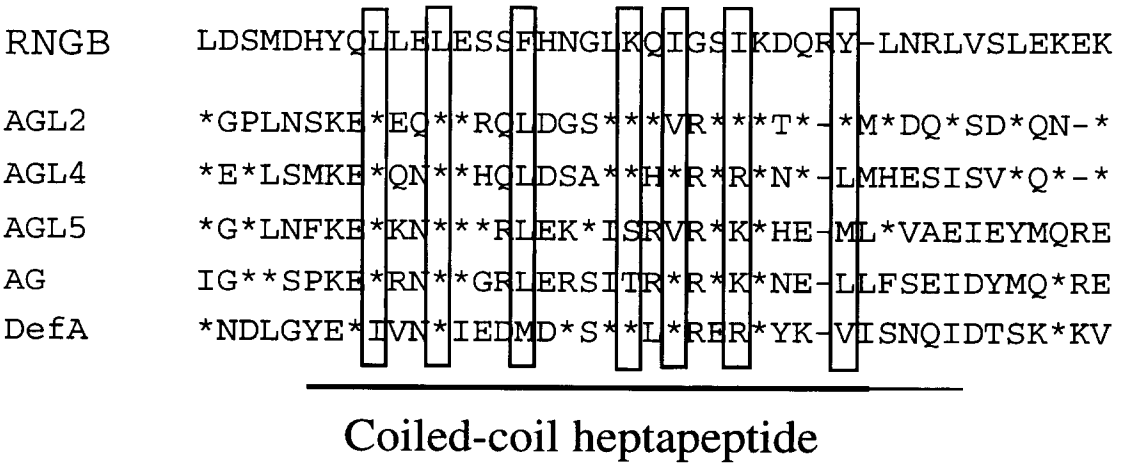


Fig. 2. (A) Sequence alignment of the Ring finger family. Supposed metal binding residues, which are absolutely conserved among family members, are boxed and conserved hydrophobic residues are indicated by a $. Amino acid insertions are indicated in parentheses. The figure is adapted from Lovering et al. [3]. (B) The alignment of plant protein K-boxes and the possible K-box homology region in the RNGB protein. A possible helix is indicated by a line below the region. Residues at positions a and d in the coiled-coil heptapeptide structure are boxed. The figure is adapted from Ma et al [15].

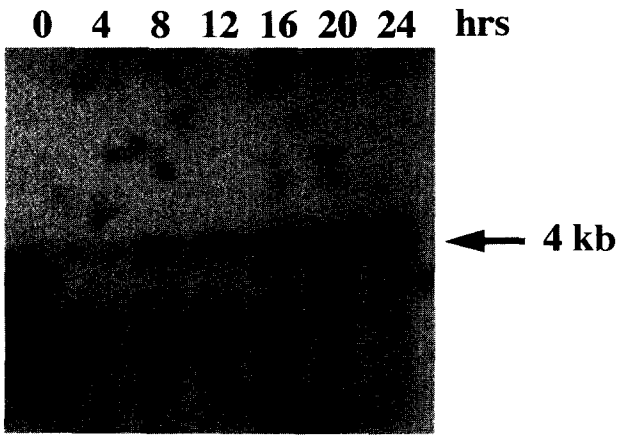


Fig. 3. Northern blot analysis of *D. discoideum* mRNA isolated from various developmental stages. Poly(A)$^+$ RNA was isolated from the stages indicated at the top of the blot. Approx. 1.5 μg of poly(A)$^+$ RNA was loaded in each lane, fractionated on a formaldehyde-containing agarose gel, and blotted onto a Hybond-N+filter. The filter was hybridised with a ^{32}P-labelled *rngB* PCR fragment (probe B in Fig. 1A) lacking any repeat sequences. The arrow indicates the *rngB* transcript, which is approx. 4 kb in length.

room temperature for 1 h with shaking, washed extensively and then incubated with 10000-fold diluted horseradish peroxidase conjugated anti-mouse IgG antibody (Bio-Rad) as above. The filter was washed and then developed using th ECL detection kit (Amersham).

For immunostaining, cells at the preculminant stage (approx. 22–24 h of development) were suspended in KK2 containing 20 mM EDTA and disaggregated by passing them through a syringe needle (25G). They were placed on a poly(L-lysine) coated slide and incubated overnight with anti-RNGB antibody, washed and incubated with FITC-conjugated anti-mouse IgG antibody and finally washed and covered with gelvatol. The cells were visualised on a confocal microscope (Bio-Rad, Model MRC1000).

3. Results

3.1. Nucleotide sequence of the rngB mRNA and deduced sequence of the RNGB protein

A *Dictyostelium* cDNA library was used in a 'one-hybrid' screen in yeast in attempt to clone the repressor which regulates stalk-specific gene expression in *Dictyostelium* [14]. The *rngB* cDNA was isolated because it directed a 10-fold higher level of expression of the reporter construct than was present in the background clones. However, in subsequent studies it was found to have only a 1.5-fold higher level than when the target DNA binding site was absent from the reporter construct. Also, we could not show specific DNA binding using RNGB protein produced by in vitro translation or in *E. coli*. Combined with the localisation evidence presented below, these data show that it is not the repressor of stalk cell differentiation but we pursued its function further by a combination of biochemical and genetic approaches.

Other *rngB* cDNA clones were derived by screening the primary filters with PCR fragments and the full-sized cDNA (rngB-F) was obtained by subcloning the *Xho*I-*Eco*RI fragment from rngB clone 8 into *Sal*I-*Eco*RI digested *rngB* clone 23 (see Fig. 1A). Sequence analysis of the *rngB* cDNA shows it to contain an open reading frame encoding a protein of 943 amino acids (Fig. 1B). The RNGB protein is predominantly hydrophilic and the carboxy-terminal half is especially hydrophilic. There are two serine-rich domains and several homopolymeric runs of asparagine and glutamine, features that are common to very many developmentally regulated *Dictyostelium* proteins.

There are two leucine (or isoleucine) heptad repeats, that might act as leucine zippers. Protein-protein interactions mediated by the zipper sequences may account for the stimulation observed in the yeast one hybrid screen. Homology searches revealed the presence of two additional, potentially important motifs near the carboxy-terminus: a RING-finger domain and a K-box homology (Fig. 2A,B). RNGB has a RING finger that fits perfectly to the consensus of such sequences (Fig. 2A). The floral homeotic proteins AGAMOUS (AG), DEFICIENS A (defA) and AGL1-6 all share a weak similarity to a portion of keratin and this consensus sequence is called a K-box [15]. There is a K-box in RNGB and it contains the characteristic coiled coil heptapeptide structure (Fig. 2B).

3.2. Analysis of the accumulation of the RNGB mRNA

Expression of the *rngB* gene is developmentally regulated. The mRNA is present in low amounts in growing cells and increases steadily during development (Fig. 3). Initially, we believed that the presence of small amounts of mRNA in growing cells explained our complete inability to generate disruptants of the *rngB* gene. The normal frequency of homologous gene disruption in *Dictysotelium* is between 10 and 90%. However, when a *rngB* disruption construct was transformed into *Dictyostelium* cells, and 300 clones were analysed by either Southern transfer or PCR for a homologous recombinant, there were no clones that showed gene disruption (data not shown). This suggested that the *rngB* gene might be essential for growth but expression of several different antisense RNA constructs of *rngB*, driven by the folic acid regulatable discoidin Iγ promoter [16], had no effect on cell growth or morphology (data not shown). It may be that there is a peculiarly low rate of recombination at the *rngB* locus or it may be that vector integration in that region is lethal for some reason unrelated to the expression of the *rngB* gene.

3.3. Analysis of the accumulation and cellular localisation of the RNGB protein

Another fact that argues against an essential role for the RNGB protein in growing cells is that the protein does not accumulate until very late in development. Accumulation was analysed immuno-histochemically, using a monoclonal antibody directed against a peptide present in the C-terminal region of the protein. Western analysis of extracts of *E. coli* cells expressing the RNGB protein under control of the T7 promoter showed a band at the expected size of 110 kDa, confirming that the antibody is directed against the RNGB protein (Fig. 4A). However, in *Dictyostelium* only cells at the very latest stages of development show a signal and the protein is only 67 kDa in size (Fig. 4B).

In order to confirm that the antibody was detecting RNGB protein in *Dictyostelium* cells, we analysed cells expressing the *rngB* gene under the control of the actin 15 promoter [17]. The actin 15 promoter is active during growth and early development and there is a band, again of 67 kDa (Fig. 4C), in cells at the slug stage (16 h of development): i.e. at a time well before the protein becomes detectable in untransformed cells (cf Fig. 4B). We conclude that the antibody specifically detects the RNGB protein and that the protein is subject to proteolytic processing.

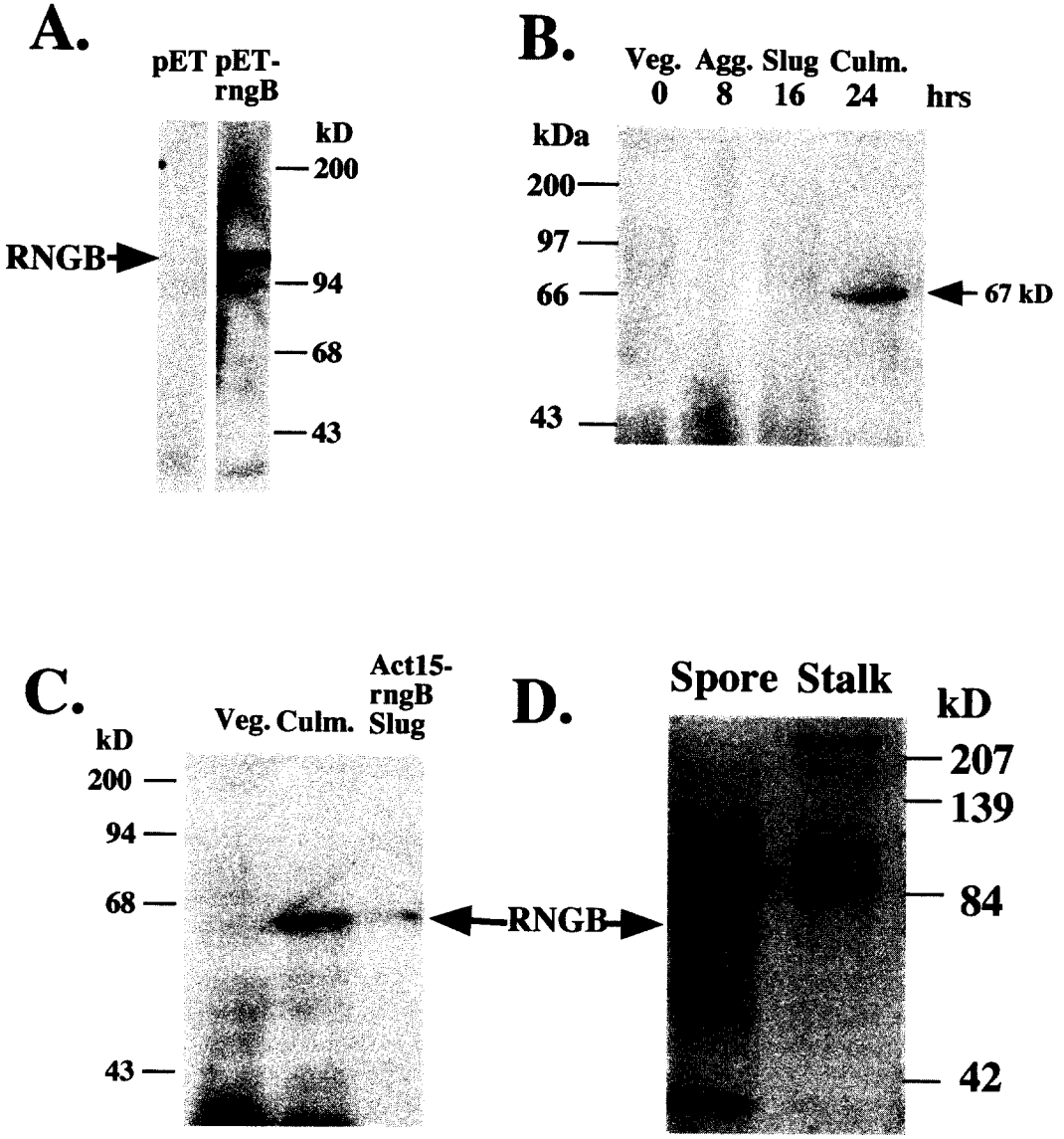


Fig. 4. Western blot analyses of the RNGB protein. (A) Expression in *E. coli*. After induction with IPTG, soluble proteins were extracted from the *E. coli* strain BL21(DE3) containing either the pET-16b (NOVAGEN) vector or pET-rngB, wherein the cDNA for RNGB falls under the control of the T7 promoter. Approx. 40 μg of proteins were loaded and separated on a 7.5% SDS-polyacrylamide gel and blotted onto a Hybond-C filter. (B) Expression in *Dictyostelium*. Total *Dictyostelium* proteins were extracted from various developmental stages indicated at the top of the blot. Approx. 50 μg of the proteins were loaded and separated on a 10% SDS-polyacrylamide gel and blotted onto a Hybond-C filter. The filter was incubated with anti-RNGB antibody and then with peroxidase conjugated anti-mouse IgG antibody. It was developed using the ECL detection kit (Amersham). (C) Expression under the control of a heterologous *Dictyostelium* promoter. Total protein was extracted from slug stage cells in which the *rngB* cDNA is transcribed under the control of the actin 15 promoter. The fragment cloned in the fusion construct derives from RngB-F, which was cleaved at a *Bam*HI site located in psD10 sequences immediately upstream of the insert and with *Xho*I, which is located at the 3′ end of the cDNA insert (Fig 1A). The rngB-8 cDNA clone contains 166nt of 5′ non-coding sequence and there is a *Xho*I site at nucleotide 87. This *Xho*I site was the position at which vector sequences were fused to cDNA sequence in the construction of rngB-F. Thus, approximately half the known 5′ non-coding region sequences are present in the fusion construct). The *Bam*H1-*Xho*I fragment was cloned into *Bgl*II-*Sal*I cleaved DIP-j, a vector that contains the actin 15 gene (R.A. Firtel, personal communication). The *Bgl*II site lies just upstream of the ATG of actin 15. Protein from the transformants was analysed in parallel with proteins isolated from vegetative cells and culminants. (D) Expression at the culmination stage. Total proteins were extracted either from spore or stalk cells of fully formed fruiting bodies. Approx. 50 μg of the proteins were loaded and separated on a 10% SDS-polyacrylamide gel and blotted onto a Hybond-C filter. The filter was analysed as above. The bands at high molecular weight and in both lanes are the result of spurious reactions with the secondary antibody. They are also present in the other analyses presented in this figure but at a much lower level relative to the RNGB signal.

Western transfer analysis on stalk and spore cells separated from fruiting bodies showed that the 67 kDa fragment from the RNGB protein is detected in the spore cells but not in stalk cells (Fig. 4D). In immuno-histochemical staining the protein is detected in the maturing spores (Fig. 5). It is not detectable in the mature spores but this could be caused by impermeability of the spore coat, preventing access of the antibody to its epitope, because it is detectable in mature spores by Western blotting (Fig. 4D). In the maturing spores the RNGB protein is present within the cytoplasm rather than the nucleus (Fig. 5). It may be associated with some organelle, as the staining appears punctate.

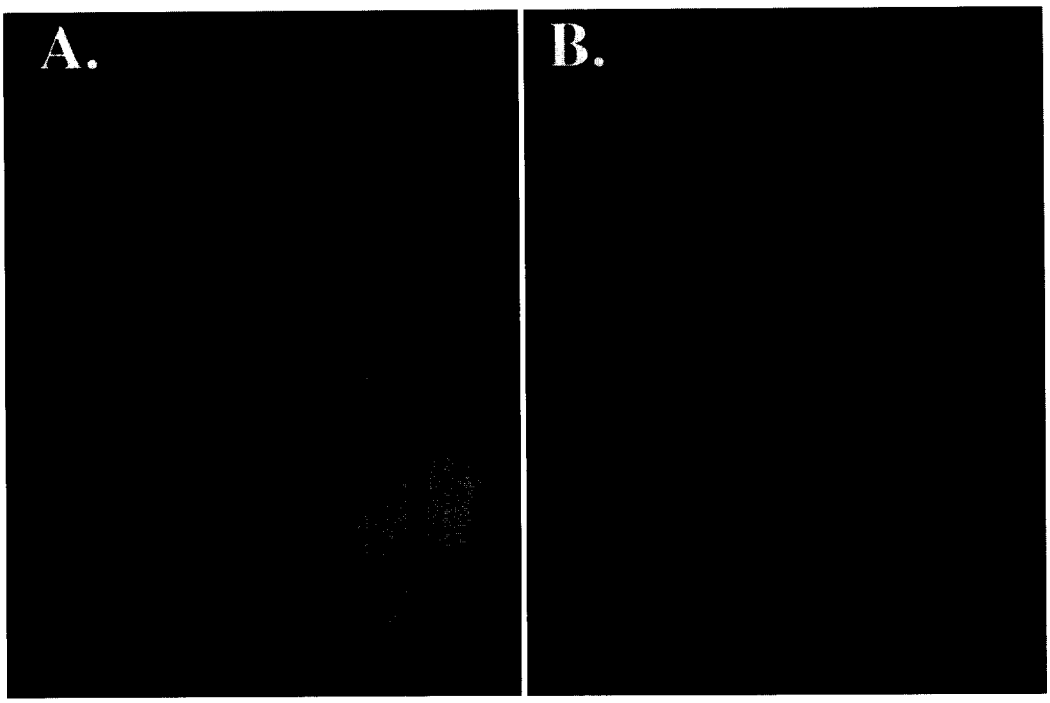


Fig. 5. Cellular localisation of the RNGB protein. Cells were dissagregated with KK2 containing 20 mM EDTA from a plate bearing a mixture of preculminant and culminant stages (approx. 22–24 h of development) and incubated either with anti-RNGB antibody (A) or with PBSA (B) followed by incubation with FITC-conjugated anti-mouse IgG antibody. Cells were analysed in the MRC1000 confocal microscope (Bio-Rad).

4. Discussion

4.1. The RNGB protein appears to be be traslationally or posttransltionally regulated

The first RING finger protein to be described was that encoded by DG17, a *Dictyostelium* gene of unknown function that is expressed during aggregation [18]. Because *Dictyostelium* genes are now routinely named using the Demerec nomenclature, we propose renaming DG17 to be rngA. We have therefore named the gene described in the present paper *rngB*. The *rngB* cDNA was isolated during a yeast one hybrid screen for the repressor that regulates stalk cell differentiation [13,14]. Detailed analysis showed that it is definitely not the repressor but, because it contains a K-box and a RING finger, we characterised it further in the expectation that it might be a transcriptional regulator with some other function.

During development the concentration of the *rngB* mRNA increases but this is not accompanied by a rise in RNGB protein concentration. The protein appears only during terminal development and it selectively accumulates in prespore cells as they are maturing into spores. Either the RNGB protein itself must become stabilised during development or, alternatively, there must be control at the translational level. The fusion gene analysis argues against control at the level of protein stability, because the actin 15-rngB construct produces authentic RNGB protein (i.e. it contains no actin sequences) and it accumulates many hours earlier than protein derived from the *rngB* gene itself. This result suggests that there is translational control of *rngB* and that it is likely to be mediated by the non-coding region *rngB* sequences that are absent from the fusion construct (see legend to Fig. 4C). Translational control is well documented in *Dictyostelium*. When cells are allowed to develop for several hours and then disaggregated a sub-set of the mRNAs dissociate from the polysomes and their translation ceases [19]. The behaviour of the RNGB protein is, however, more analogous to the situation observed for the *drsA* mutant, which accumulates discoidin I mRNA without accumulating the protein [20].

4.2. The RNGB protein is cytosolic and is subject to post-translational cleavage

The RNGB protein is proteolytically cleaved, perhaps as part of its processing. It is present within the cytoplasmic and this is further confirmation that it is not, as was initially thought, a transcriptional regulator. In all the known cytoplasmic RING finger proteins the finger is located near the C-terminus but it is located near the N-terminus in the nuclear proteins [1]. The RNGB conforms to this pattern. The spacing of residues in the finger is similar to family proteins involved in inhibition of apoptosis (IAP), although there is no IAP consensus in the RNGB protein [21].

The function of the RNGB protein remains obscure but it will be a useful protein marker of spore maturation. Until now the only marker of this process was the product of the *spiA* gene, a protein that is required for maintaining integrity under conditions of osmotic shock [22].

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