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Heteroplasmic mitochondrial disease in *Dictyostelium discoideum*

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ABSTRACT

The bewildering complexity of the relationship between genotype and phenotype in human mitochondrial diseases has delayed an understanding of the related cytopathological mechanisms. To explore the relationship between mitochondrial dysfunction in *Dictyostelium discoideum* and the related cytopathologies, we determined whether the phenotypic outcomes were similar regardless of which *D. discoideum* mitochondrial gene was targeted for disruption. The disruption of the mitochondrial genes resulted in a similar pattern of phenotypes to those caused by other mitochondrial defects. These include impairment of phototaxis, multicellular development and growth on plates and in liquid medium. As the reduced growth rates could have been due to defective phagocytic or macropinocytic nutrient uptake, these processes were tested but found to be unaffected. Since mitochondria have been associated with *Legionella* pathogenesis of human macrophages, it was also determined if mitochondrially diseased *Dictyostelium* strains were better or worse than healthy cells at supporting the growth of *Legionella pneumophila*. The results revealed that the mitochondrially diseased strains supported greater *L. pneumophila* growth than the wild type *Dictyostelium* strain (AX2). Quantitative Northern blotting showed a significant reduction in the level of expression of the entire mitochondrial genome, regardless of which mitochondrial gene was targeted for disruption, suggesting a generalized deficiency in mitochondrial gene expression and function. The phenotypic outcomes were the same as those shown previously to result from chronic hyperactivity of the energy-sensing protein kinase, AMPK, after knockdown of mitochondrial chaperonin 60.

1. Introduction

Mutations in mitochondrial or nuclear genes that encode essential mitochondrial proteins are associated with an array of human mitochondrial diseases. Unfortunately the complexities of mammalian developmental and mitochondrial biology have meant that the clinical outcomes of mitochondrial disease are not predictable from the genetic defect causing them. Individuals with the same mutation can exhibit very different signs and symptoms while different genetic defects can cause very similar pathologies. Depending on which tissues are affected most in a given individual, this leads to a bewildering complexity in mitochondrial diseases which exhibit a very broad range of possible symptoms. These symptoms can include epilepsy, stroke-like episodes, muscle weakness, ataxia, exercise intolerance, parkinsonism, blindness, deafness, diabetes, kidney malfunction, heart conditions and various combinations of these clinical manifestations [1–4]. Examples of mitochondrial diseases include Myoclonic Epilepsy with Ragged-Red Fibers (MERRF); Mitochondrial Encephalomyopathy, Lactic Acidosis and Stroke-like episodes (MELAS); Leber's Hereditary Optic Neuropathy (LHON); Neuropathy, Ataxia and Retinitis Pigmentosa (NARP); and Leigh syndrome (LS) [1,4].

To effectively treat mitochondrial diseases a clear understanding of the associated disease mechanisms is necessary. In recent years *Dictyostelium* has emerged as a valuable model organism to investigate the molecular basis for cytopathologies associated with mitochondrial dysfunction [5–13]. An advantage of the *Dictyostelium* model is that it offers a tractable microbial system in which a wide range of readily assayed, reproducible phenotypes can been studied. In mitochondrial disease studies these have included phototaxis, thermotaxis, chemotaxis, growth in suspension and on bacterial lawns, phagocytosis, macropinocytosis, multicellular morphogenesis and susceptibility to the intracellular bacterial pathogen *Legionella pneumophila* [7,9]. Each of these phenotypes is the endpoint of regulatory signal transduction pathways whose functions may be disturbed as a result of mitochondrial dysfunction. Indeed it was proposed in the earliest studies that the primary cytopathological effect of mitochondrial disease in *D. discoideum* is dysregulation of cellular signal transduction pathways [11,13].

We previously generated mitochondrial dysfunction either by disruption of the large rRNA subunit gene (*rnl*) in a subset of the

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mitochondrial genomes (a state referred to as heteroplasmy) [13] or by antisense inhibition of expression of chaperonin 60, an essential nuclear-encoded mitochondrial protein [11]. The former would impair protein synthesis in the subpopulation of mitochondria in which *rnl* was disrupted, while the latter would reduce the protein-folding capacity of the mitochondria. In both cases, photo- and thermotaxis, growth in liquid medium and multicellular development were impaired, prompting the suggestion that the phenotypic outcomes of mitochondrial dysfunction in *Dictyostelium* are the same, regardless of the underlying genetic cause. In the work reported here, we test this hypothesis further by examining the phenotypic consequences of heteroplasmic disruption of eight further mitochondrial genes distributed around the mitochondrial genome [14]. These genes included some that encode subunits of respiratory chain enzymes, *atp1*, *atp6*, *cob*, *cox3*, *nad2* and *nad5*, as well as the open reading frames *ORF1740* and *ORF796*. *ORF1740* encodes a ribosomal protein, S3 C-terminal domain-containing protein, whereas the function of the *ORF796* product is unknown.

After the original studies of mitochondrial dysfunction in *Dictyostelium*, a wider range of phenotypes was investigated in chaperonin 60 antisense-inhibited strains [7,9] and in knockout mutants lacking a novel mitochondrial protein, MidA [12,15]. Some phenotypes were common to both kinds of mutant, namely defective phototaxis, thermotaxis, growth in suspension and on plates. However the chaperonin 60 antisense-inhibited strains exhibited normal phagocytosis and macropinocytosis rates [7], while the *midA*$^{-}$ mutant was defective for both of these phenotypes [12]. Here we show that the phenotypic outcomes of disrupting any of 9 different mitochondrial genes are similar to those arising from chaperonin 60 antisense inhibition and different from those reported in the *midA* knockout mutant.

The *Dictyostelium* mitochondrial genome is transcribed unidirectionally from a single promoter and the transcript is processed to produce the mature RNA products [16]. We show here that regardless of which gene is targeted, heteroplasmic mitochondrial gene disruption causes a coordinate reduction in expression of transcripts encoded near the start, middle and end of the primary transcript i.e. expression of the entire mitochondrial genome is depressed. This generalized mitochondrial dysfunction contrasts with the specific impairment of Complex I activity observed when MidA is knocked out [15], but is akin to the general effects on mitochondrial protein synthesis or folding expected in *rnl* disruptants or chaperonin 60 knockdown strains.

2. Methods

2.1. Plasmid constructs and Dictyostelium strains

All experiments were performed with *D. discoideum* parental strain AX2 [30] and mutants created from it. *D. discoideum* mutant strains were created by heteroplasmic disruption of nine mitochondrial genes using the previously described strategy [13]. The approach is based on homologous recombination between the mitochondrial genome and a circular plasmid molecule containing an insert of 500–1000 bp of the targeted gene. Cells were transformed with the circular plasmid construct and stable transformants were selected on the basis of G418 [geneticin (Promega Corporation, Annandale, NSW, Australia)] resistance expressed under the control of a constitutive chromosomal gene promoter in the vector. The desired, targeted insertions into the mitochondrial genome are accompanied by multiple, independent insertions at nontargeted locations in the nuclear genome [13]. Each of these random chromosomal insertions contains scores to hundreds of tandemly duplicated copies of the construct that are produced by rolling circle replication during the insertion process [20]. In such targeted disruption experiments, the desired insertion into the targeted gene occurs only in a minority of transformants. In this work we therefore screened the transformants initially for mitochondrial gene disruptions on the basis of the phototaxis-deficient phenotype previously shown to be characteristic of mitochondrial dysfunction. This was followed by Southern blotting to verify the presence of plasmid insertions into the mitochondrial genome as previously [13] (see Section 3). The plasmid constructs and mutant strains isolated using them are listed in Table 3.

Chaperonin 60 antisense mutants (HPF406-418) were described previously by Kotsifas et al. [11]. The mitochondrial, large ribosomal RNA gene (*rnl*) mutants (HPF266–270) were described previously by Wilczynska et al. [13].

2.2. Culture conditions

Cells grown axenically were cultured in HL-5 liquid medium [30] supplemented with 100 μg/ml ampicillin (Roche Diagnostics Australia Pty. Ltd., Castle Hill, NSW Australia), 20 μg/ml streptomycin (Boehringer Mannheim Australia Pty. Ltd., Castle Hill, NSW Australia) and 10 μg/ml tetracycline (Sigma–Aldrich Pty. Ltd., Castle Hill, NSW Australia). Strains were also grown on bacterial lawns prepared from *Klebsiella aerogenes* on SM agar. As a selective marker, G418 (geneticin) (20 ug/ml) was added to the growth media for all transformants during subculturing. However, for phenotypic studies antibiotics were excluded from media to avoid any possible antibiotic-associated effects.

2.3. Phototaxis

A small quantity of amoebae was scraped from the edges of *Dictyostelium* colonies on *K. aerogenes* lawns by toothpick and transferred to non-nutrient charcoal agar plates [1.0% agar (Oxoid Australia Pty. Ltd., Adelaide, South Australia, Australia), 0.5% activated charcoal (Sigma–Aldrich)] [18,31]. After incubation with a lateral light source for 24–48 h, slugs and slime trails were transferred to clear polyvinyl chloride (PVC) discs. The discs were stained for 5 min with Coomassie blue (Sigma–Aldrich) [31] and rinsed gently with running tap water. To perform quantitative analysis, start and end points of the stained trails were digitised and stored as *x*, *y* coordinates through use of the Summagraphics 120 digitising tablet connected to a SUN workstation (SUN Microsystems, Santa Clara, CA, USA), and analysed using directional statistics based on the von Mises or circular normal distribution [18].

2.4. Pulsed field gel electrophoresis and Southern blot analysis

For PFGE the cells were prepared according to the method of Cox et al. [32]. The cells were washed twice in ice-cold phosphate buffer [17 mM Na/K phosphate (Ajax Chemicals Pty. Ltd., Auburn, NSW, Australia), pH 6.0] and then mixed with an equal volume of 2% InCert® low melting (LM) point agarose (FMC BioProducts, Rockland, ME, USA) which was precooled to 39 °C. The mixture (final density = (4–5) × 10^8 cells/ml) was then quickly pipetted into prewarmed moulds (~250 μl for each plug). The solidified plugs (~30 μl) were incubated in digestion buffer [0.5 M EDTA (Sigma–Aldrich), pH 8.0; 2% sodium lauroyl-sarcosinate (Sigma–Aldrich); 2 mg/ml Proteinase K (Gibco BRL, Gaithersburg, MD, USA)] for 48 h at 50 °C.

A series of washes were performed before restriction enzyme digestion of DNA. The agarose plugs were repeatedly washed in TE buffer containing 1 mM phenylmethylsulfonyl fluoride [PMSF (Sigma™)] to inactivate Proteinase K. The plugs were then repeatedly washed in TE for 30 min at RT to remove the PMSF and any remaining N-Lauroylsarcosine (Sigma–Aldrich). The