ABOUT JCBG

The Journal of Cell Biology and Genetics (JCBG) is published monthly (one volume per year) by Academic Journals.

Journal of Cell Biology and Genetics (JCBG) is an open access journal that provides rapid publication (monthly) of articles in all areas of the subject such as Cell division, Cellular respiration, Cellular respiration, Chemosynthesis etc. The Journal welcomes the submission of manuscripts that meet the general criteria of significance and scientific excellence. Papers will be published shortly after acceptance. All articles published in JCBG are peer-reviewed.

Submission of Manuscript

Submit manuscripts as e-mail attachment to the Editorial Office at: jcbg.journal@gmail.com. A manuscript number will be mailed to the corresponding author shortly after submission.

For all other correspondence that cannot be sent by e-mail, please contact the editorial office (at jcbg.journal@gmail.com).

The Journal of Cell Biology and Genetics will only accept manuscripts submitted as e-mail attachments.

Please read the Instructions for Authors before submitting your manuscript. The manuscript files should be given the last name of the first author.
Editors

Dr. Hehai Wang
*University of Arkansas,*
Fayetteville, AR.

Dr. Jaffar Ali
*King Fahad Medical City (KFMC),*
Riyadh,
*Kingdom of Saudi Arabia*

Dr. Hasan Turkez
*Division of General Biology,*
*Faculty of Science,*
*Ataturk University,*
*Erzurum, Turkey.*
Editorial Board

Dr. Qurratulain Hasan
Senior Scientific Consultant, Genetics & Molecular Biology,
Department of Genetics,
Bhagwan Mahaveer Medical & Research Centre,
AC Guards, Hyderabad, India.

Dr. Gyanendra Singh
Stanley S. Scott Cancer Center,
School of Medicine,
Louisiana State University Health Sciences Center,
New Orleans, LA 70112, USA.

Dr. Viktor R. Drel
Research Assistant Professor,
Department of Biochemistry,
Ivan Franko National University of Lviv,
Grushevskogo Str. 4, 79005, Lviv, Ukraine.
Instructions for Author

Electronic submission of manuscripts is strongly encouraged, provided that the text, tables, and figures are included in a single Microsoft Word file (preferably in Arial font).

The cover letter should include the corresponding author’s full address and telephone/fax numbers and should be in an e-mail message sent to the Editor, with the file, whose name should begin with the first author’s surname, as an attachment.

Article Types
Three types of manuscripts may be submitted:

Regular articles: These should describe new and carefully confirmed findings, and experimental procedures should be given in sufficient detail for others to verify the work. The length of a full paper should be the minimum required to describe and interpret the work clearly.

Short Communications: A Short Communication is suitable for recording the results of complete small investigations or giving details of new models or hypotheses, innovative methods, techniques or apparatus. The style of main sections need not conform to that of full-length papers. Short communications are 2 to 4 printed pages (about 6 to 12 manuscript pages) in length.

Reviews: Submissions of reviews and perspectives covering topics of current interest are welcome and encouraged. Reviews should be concise and no longer than 4-6 printed pages (about 12 to 18 manuscript pages). Reviews are also peer-reviewed.

Review Process
All manuscripts are reviewed by an editor and members of the Editorial Board or qualified outside reviewers. Authors cannot nominate reviewers. Only reviewers randomly selected from our database with specialization in the subject area will be contacted to evaluate the manuscripts. The process will be blind review. Decisions will be made as rapidly as possible, and the journal strives to return reviewers’ comments to authors as fast as possible. The editorial board will re-review manuscripts that are accepted pending revision. It is the goal of the JCBG to publish manuscripts within weeks after submission.

Regular articles
All portions of the manuscript must be typed double-spaced and all pages numbered starting from the title page.

The Title should be a brief phrase describing the contents of the paper. The Title Page should include the authors’ full names and affiliations, the name of the corresponding author along with phone, fax and E-mail information. Present addresses of authors should appear as a footnote.

The Abstract should be informative and completely self-explanatory, briefly present the topic, state the scope of the experiments, indicate significant data, and point out major findings and conclusions. The Abstract should be 100 to 200 words in length. Complete sentences, active verbs, and the third person should be used, and the abstract should be written in the past tense. Standard nomenclature should be used and abbreviations should be avoided. No literature should be cited. Following the abstract, about 3 to 10 key words that will provide indexing references should be listed.

A list of non-standard Abbreviations should be added. In general, non-standard abbreviations should be used only when the full term is very long and used often. Each abbreviation should be spelled out and introduced in parentheses the first time it is used in the text. Only recommended SI units should be used. Authors should use the solidus presentation (mg/ml). Standard abbreviations (such as ATP and DNA) need not be defined.

The Introduction should provide a clear statement of the problem, the relevant literature on the subject, and the proposed approach or solution. It should be understandable to colleagues from a broad range of scientific disciplines.

Materials and methods should be complete enough to allow experiments to be reproduced. However, only truly new procedures should be described in detail; previously published procedures should be cited, and important modifications of published procedures should be mentioned briefly. Capitalize trade names and include the manufacturer’s name and address. Subheadings should be used. Methods in general use need not be described in detail.
Results should be presented with clarity and precision. The results should be written in the past tense when describing findings in the authors’ experiments. Previously published findings should be written in the present tense. Results should be explained, but largely without referring to the literature. Discussion, speculation and detailed interpretation of data should not be included in the Results but should be put into the Discussion section.

The Discussion should interpret the findings in view of the results obtained in this and in past studies on this topic. State the conclusions in a few sentences at the end of the paper. The Results and Discussion sections can include subheadings, and when appropriate, both sections can be combined.

The Acknowledgments of people, grants, funds, etc should be brief.

Tables should be kept to a minimum and be designed to be as simple as possible. Tables are to be typed double-spaced throughout, including headings and footnotes. Each table should be on a separate page, numbered consecutively in Arabic numerals and supplied with a heading and a legend. Tables should be self-explanatory without reference to the text. The details of the methods used in the experiments should preferably be described in the legend instead of in the text. The same data should not be presented in both table and graph form or repeated in the text.

Figure legends should be typed in numerical order on a separate sheet. Graphics should be prepared using applications capable of generating high resolution GIF, TIFF, JPEG or Powerpoint before pasting in the Microsoft Word manuscript file. Tables should be prepared in Microsoft Word. Use Arabic numerals to designate figures and upper case letters for their parts (Figure 1). Begin each legend with a title and include sufficient description so that the figure is understandable without reading the text of the manuscript. Information given in legends should not be repeated in the text.

References: In the text, a reference identified by means of an author’s name should be followed by the date of the reference in parentheses. When there are more than two authors, only the first author’s name should be mentioned, followed by ‘et al’. In the event that an author cited has had two or more works published during the same year, the reference, both in the text and in the reference list, should be identified by a lower case letter like ‘a’ and ‘b’ after the date to distinguish the works.

Examples:

Abayomi (2000), Agindotan et al. (2003), (Kelebeni, 1983), (Usman and Smith, 1992), (Chege, 1998; 1987a,b; Tijani, 1993,1995), (Kumasi et al., 2001)

References should be listed at the end of the paper in alphabetical order. Articles in preparation or articles submitted for publication, unpublished observations, personal communications, etc. should not be included in the reference list but should only be mentioned in the article text (e.g., A. Kingori, University of Nairobi, Kenya, personal communication). Journal names are abbreviated according to Chemical Abstracts. Authors are fully responsible for the accuracy of the references.

Examples:


Short Communications

Short Communications are limited to a maximum of two figures and one table. They should present a complete study that is more limited in scope than is found in full-length papers. The items of manuscript preparation listed above apply to Short Communications with the following differences: (1) Abstracts are limited to 100 words; (2) instead of a separate Materials and Methods section, experimental procedures may be incorporated into Figure Legends and Table footnotes; (3) Results and Discussion should be combined into a single section.

Proofs and Reprints: Electronic proofs will be sent (e-mail attachment) to the corresponding author as a PDF file. Page proofs are considered to be the final version of the manuscript. With the exception of typographical or minor clerical errors, no changes will be made in the manuscript at the proof stage.
Fees and Charges: Authors are required to pay a $550 handling fee. Publication of an article in the Journal of Cell Biology and Genetics is not contingent upon the author's ability to pay the charges. Neither is acceptance to pay the handling fee a guarantee that the paper will be accepted for publication. Authors may still request (in advance) that the editorial office waive some of the handling fee under special circumstances.

All rights Reserved. In accessing this journal, you agree that you will access the contents for your own personal use but not for any commercial use. Any use and or copies of this Journal in whole or in part must include the customary bibliographic citation, including author attribution, date and article title.

Submission of a manuscript implies: that the work described has not been published before (except in the form of an abstract or as part of a published lecture, or thesis) that it is not under consideration for publication elsewhere; that if and when the manuscript is accepted for publication, the authors agree to automatic transfer of the copyright to the publisher.

Disclaimer of Warranties

In no event shall Academic Journals be liable for any special, incidental, indirect, or consequential damages of any kind arising out of or in connection with the use of the articles or other material derived from the JCBG, whether or not advised of the possibility of damage, and on any theory of liability.
This publication is provided "as is" without warranty of any kind, either expressed or implied, including, but not limited to, the implied warranties of merchantability, fitness for a particular purpose, or non-infringement.
Descriptions of, or references to, products or publications does not imply endorsement of that product or publication. While every effort is made by Academic Journals to see that no inaccurate or misleading data, opinion or statements appear in this publication, they wish to make it clear that the data and opinions appearing in the articles and advertisements herein are the responsibility of the contributor or advertiser concerned. Academic Journals makes no warranty of any kind, either express or implied, regarding the quality, accuracy, availability, or validity of the data or information in this publication or of any other publication to which it may be linked.
<table>
<thead>
<tr>
<th>ARTICLES</th>
</tr>
</thead>
<tbody>
<tr>
<td>Research Articles</td>
</tr>
<tr>
<td><strong>Constitutive activation of AMP-activated protein kinase (AMPK) propel mitochondrial biogenesis</strong></td>
</tr>
<tr>
<td>Pankaj Chavhan and Amir Dhamani</td>
</tr>
</tbody>
</table>
Constitutive activation of AMP-activated protein kinase (AMPK) propel mitochondrial biogenesis

Paul B. Bokko¹,²*, Afsar U. Ahmed¹,³ and Paul R. Fisher¹

¹School of Life Sciences, La Trobe University, Bundoora, VIC 3086, Australia.
²Department of Veterinary Surgery and Theriogenology, University of Maiduguri, PMB 1069, Nigeria.
³Monash Medical Research Institute, Monash University, Clayton, VIC 3800 Australia.

Mitochondrial biogenesis is primarily a critical adaptation aimed to balance an increased workload in an attempt to maintain appropriate body perfusion. Until recently, the signaling mechanisms responsible for this response are poorly understood. To examine the role of AMP-activated protein kinase (AMPK), an evolutionarily conserved fuel sensor, in mitochondrial biogenesis, we used constitutively active and antisense inhibition genetic tools in Dictyostelium discoideum. Constitutive activation of AMPK culminated in mitochondrial proliferation and elevated ATP generation; this became marked with higher plasmid copies. Antisense inhibition of AMPK yielded non–significant decrease in the mitochondrial content at low levels. However, the more severe the antisense inhibition, the more significant the diminution of AMPK function, resulting in the more apparent decrease in the Advanced Technology Program (ATP) and mitotracker fluorescence. This finding provides direct genetic evidence that AMPK plays a significant role in ameliorating the effects of cellular energy deficit through mitochondrial proliferation. Thus, the constitutive activation of AMPK initiates signalling to downstream targets. The result perturbation of these pathways would culminate in the mitochondrial biogenesis. Taken together, these findings show the constitutive activation of AMPK propels in vivo mitochondrial biogenesis and ATP generation in D. discoideum as in other organisms.

Key words: Antisense, constitutive activation, Dictyostelium discoideum, mitochondrial mass, plasmid copies.

INTRODUCTION

Mitochondrial biogenesis is a critical adaptation to chronic energy deprivation such as chronic metabolic stress and long-term exercise, rather than in phase with the cell cycle (Zong et al., 2002; Ventura et al., 2008). During increased energy demands, imbalance between rates of adenosine triphosphate (ATP) synthesis and consumption in the mitochondria would lead to a deleterious drop in energy-rich phosphate compounds (Goffart et al., 2004). Moreover, the primary indicator of altered energy production in the cytosol is the [ATP][ADP]/[Pi] ratio (Hardie and Hawley, 2001). The problem may even be aggravated if there are defects in mitochondrial function...
itself (Bokko et al., 2007). The energy depletion becomes physiologically notable especially during high workload, and this leads to the induction of compensatory reactions and adaptations that result in an increase in the maximal capacity to generate ATP (Wakerhage and Woods, 2002). The accuracy of mitochondrial biogenesis relies on the spatiotemporally coordinated synthesis, import and correct assembly of about 1,100 nuclear encoded proteins (Ventura et al., 2008; Scarpulla et al., 2012). The precursor proteins are escorted by molecular chaperones, imported and refolded into intramitochondrial proteins and to specific mitochondrial compartments (Hermann and Neupert, 2000). Other proteins encoded by mitochondrial DNA (mtDNA) necessary for the components of the electron transport chain, mitochondrial replication, fusion and fission must also be tightly synchronized to ensure proper mitochondrial function and shape; while striving to meet tissue energy requirements (Ventura et al., 2008). The ability to replicate enables proper organization of the mitochondrial network during biogenesis (Hermann et al., 1998; Chan, 2006). Thus, mitochondrial biogenesis is accompanied by variations in mitochondrial size, number and mass (Ventura et al., 2008; Scarpulla et al., 2012). Conversely, mitochondria can also undergo fusion or become inactive when energy use is low (Hermann et al., 1998; Chan, 2006).

Recent advances in molecular biology have started to elucidate the transcriptional events governing mitochondrial biogenesis. For instance, acute activation of AMPK in muscle helps defend against energy deficiency by promoting increased glucose transport and fatty acid oxidation through increased GLUT4 translocation (Ihlemann et al., 1999; Hayashi et al., 2000; Bergeron et al., 2001). Prolonged AMPK activation coordinately triggers cell adaptation to energy stress by translating diverse physiological/metabolic perturbations into an increased capacity to generate energy via the expression of key transcriptional regulator genes of mitochondrial biogenesis (Bergeron et al., 2001). A particular significance is the expression of the peroxisome proliferator-activated receptor-γ (PPAR-γ) coactivator 1α (PGC-1α)/PPARα (Puigserver et al., 1998; Wu et al., 1999; Lin et al., 2002; Goffart et al., 2004) and calcium/calmodulin-dependent protein kinase IV (CaMK IV) (both master regulators of mitochondrial biogenesis) (Hawley et al., 1995; Wu et al., 2002).

Coactivation of PGC-1α and nuclear respiratory factors (NRF-1 and NRF-2) cause the induction of the mitochondrial transcription factor A (mTFA) (Puigserver et al., 1998; Wu et al., 1999; Lin et al., 2002). The mTFA in turn upregulates the expression of nuclear and mitochondrial genes encoding mitochondrial proteins (Gulick et al., 1994; Wu et al., 1999; Bergeron et al., 2001; Lehman and Kelly, 2002; Vega et al., 2000). The outcome is transcription and replication of key mitochondrial enzymes (Clayton, 1992; Winder et al., 2000), mitochondrial biogenesis to increase mitochondrial oxidative capacity (Williams et al., 1986; Zong et al., 2002) and ATP generation (Xia et al., 1997; Bergeron et al., 2001). Expression of PGC-1α in C2C13 myoblasts cells (Wu et al., 1999), the heart (Lehman et al., 2000; Michael et al., 2001; Lehman and Kelly, 2002; Suzuki et al., 2007) and in skeletal muscle of transgenic mice stimulates mitochondrial biogenesis leading to a dramatic increase of mitochondrial mass and size in neonatal mice and mitogenesis in adult mice (Michael et al., 2001; Lin et al., 2002). The action of PGC-1α in turn is controlled by the coordinated actions of myocyte enhancer factor-2 (MEF2) transcription factor and class II histone deacetylases (HDACs) regulatory pathway via two MEF2-binding sites on the upstream/promoter region of PGC-1α (McKinnie et al., 2001; Czubryt et al., 2003; Black and Olson, 1998). The MEF2 through PGC-1α drive mitochondrial enzyme expression, growth and mitochondrial energy production (Czubryt et al., 2003). Prolonged MEF2 function activates HDACs, thereby negatively regulating the expression of PGC-1α (Grozienger and Schreiber, 2000; McKinsey et al., 2001). Conversely, inactivation of HDACs kinases derepresses PGC-1α expression, resulting in increased mitochondrial biogenesis (Czubryt et al., 2003).

Furthermore, chronic administration into nonexercising muscles of β-guanidinopropionic acid (GPA) (depletes phosphocreatine and ATP) (Bergeron et al., 2001) or 5-aminoimidazole-4-carboxamide-1-β-d-ribofuranoside (AICAR) (Corton et al., 1995; Reznick and Shulman, 2006) or metformin (Liu et al., 2006) increases the expression of CaMK IV and PGC-1α in skeletal muscle (Bergeron et al., 2001; Zong et al., 2002). In addition, NRF-1 binding activity, δ-ALAS mRNA expression, cytochrome c protein expression, key mitochondrial enzymes and mitochondrial content are also upregulated. Conversely, GPA treatment of transgenic mice expressing dominant-negative mutant of AMPK expression (DN-AMPK) in muscle did not induce the expression of signaling molecules/pathways like the PGC-1α and CaMK IV or exhibit mitochondrial biogenesis (Woods et al., 2000; Zong et al., 2002). Recently, expression of endothelial nitric oxide synthase (eNOS) increased mitochondrial DNA content, cytochrome c and cytochrome c-oxidase IV protein expression levels, and PGC-1α, NRF-1, and mtTFA mRNA expression (Nisoli et al., 2003), and consequently, mitochondrial biogenesis. Though, it has been known for decades that physical activity is associated with increased mitochondrial content, it is only known recently that some of the critical factors involved in the regulation of mitochondrial biogenesis have been identified. AMPK is an attractive potential candidate, and its role in this process has been advocated by recent correlative studies demonstrating that AMPK activation is associated with increased
mitochondrial enzyme content (Winder et al., 2000) and mitochondrial biogenesis in rats (Bergeron et al., 2001). In earlier report, AMPK phenocopied mitochondrial dysfunction and signal transduction dose dependently. Given AMPK functions as key regulator of energy metabolism, the study centred on assessment of its role in mitochondrial biogenesis. Here we report that constitutive activation of AMPK propel mitochondrial biogenesis in Dictyostelium discoideum.

MATERIALS AND METHODS

Many molecular biological techniques were used as described by Ausubel et al. (1995). Sequence analyses, alignments and database searches were conducted using web-based software through dictyBase (http://www.dicybase.org/blast), ExPASy (Expert Protein Analysis System) site (http://www.expasy.org) and at the Australian Genome Research Facility (www.agrf.org.au).

Dictyostelium discoideum: Strains and culture conditions

All experiments were conducted with D. discoideum strain AX2 (Watts and Ashworth, 1970) and strains derived from it (Bokko et al., 2007). Each strain carried multiple copies of the following plasmids: (1) pPROF362 (AMPKα subunit antisense inhibition construct) in HPF456 to 462; (2) pPROF361 (corresponding RNA control construct) in HPF466 to 468; or (3) pPROF 392 (AMPKα antisense overexpression construct) in HPF434 to 442 strains. All strains were isolated using the Ca(PO4)2/DNA coprecipitation method (Nellen et al., 1984) and selected as isolated, independent colonies on Micrococcus luteus lawns on standard medium (SM) agar supplemented with 20 μg/ml geneticin (G-418) (Promega Corporation, Madison, WI). Cells were cultured in axenic medium (HL-5) supplemented with 100 μg/ml ampicillin and 20 μg/ml streptomycin or on Klebsiella aerogenes lawns on SM agar. The selective agent geneticin (20 μg/ml) was added to HL5 medium for all strains.

Gene cloning and sequence analysis

AMPK cloning strategies and vectors were as described previously (Bokko et al., 2007). The 2.6 kb snfA gene (Sung et al., 1999; EMBL/GenBank ID AF118151) encoding the AMPK α subunit was amplified from genomic DNA template using the gene-specific primers PAMKα

P5

TTACCAGATCCGACACTACGATCTCCATGCCTACCAATATTTGCC

TT-3' and PAMKR1

5'-TCTAGACTCGAGTTAATCTACATAATATATGTAATATGTTCA

ACC-3' and cloned into pZerO2 (Invitrogen, Carlsbad, CA) for further studies. A constitutively active AMPK α subunit was generated by substituting the catalytic domain of the AMPK α subunit DNA probe (pPROF392) with an expression construct of the constitutively active AMPK α subunit (Bokko et al., 2006). The catalytic domain comprising 1140 bp cDNA fragment designated AMPK α380 was cloned into pZerO2 template (pPROF392) and subsequently transformed into the strain carrying multiple copies of the following sequences: (1) the catalytic domain of AMPK α subunit antisense (pPROF361) and sense (pPROF362) constructs, or (2) the catalytic domain of the AMPK α subunit constitutively active, truncated AMPK α subunit (pPROF392).

Creation of the AMPK α subunit antisense/sense constructs

Antisense inhibition offers partial inhibition of expression of essential genes producing sublethal phenotypes. Plasmid constructs for expression of antisense RNA and the corresponding sense RNA control were created from pPROF348 template by amplifying a fragment using primers PAMPKF1 (5'-TCTAGAATTCCTATGATGAAAAAGATTAGAGA-3') and PAMPKR1 (5'-TCTAGATTCTCCATGCTATTTGGAAGGTTA-3'), cloned into XbaI site of the pZerO2 TM-2. The DNA fragment(s) were subcloned into the EcoRI site of the Dictyostelium expression vector, qDNeo2 (Xie et al., 1997) using either orientations. The result of AMPK α subunit sense (pPROF361) and antisense (pPROF362) plasmid express 1172 bp of the AMPK α antisense RNA or the corresponding sense RNA control. Both fragments would be expressed under the control of the Dictyostelium actin 6 promoter. AMPK α subunit DNA fragments cloned into appropriate plasmids were submitted to the Australian Genome Research Facility (AGRF), Brisbane, Australia for sequencing.

Determination of plasmid copies in strains

The plasmid copies in the various strains were determined by Southern blotting using enhanced chemiluminescence measurements in a fluoroimager (Storm 860 TM) (Amersham Biosciences, Castle Hill, Sydney, Australia). Genomic DNA preps were extracted from AX2 and each strain. Each strain was digested with EcoRI, separated by gel electrophoresis, blotted onto nylon membrane and probed with fluorescein labelled AMPK α subunit DNA as well as Gβ–protein DNA probe(s) in combination with antifluorescein alkaline peroxidase-conjugated antibody. The plasmid copies were quantitated using the ImageQuan tools (TLv2003.03) program; computed as the ratio of the RFU values of respective strain to the RFU values of AX2.

Estimation of RNA expression in strains

RNA was extracted from each strain bearing plasmid vector expressing the catalytic domain of the AMPK α subunit (α380) (pPROF392) or an antisense/sense RNA complementary as part of the catalytic domain of the AMPK α subunit (pPROF362/pPROF361). Northern blotting analysis was performed by blotting nucleic acids onto nylon membrane and probed with DIG-labelled AMPK α subunit DNA probe to determine RNA expression levels in the strains.

Mitochondrial mass by fluorescence microscopy using Mitotracker green or red

Mitochondrial mass was evaluated to investigate mitochondrial biogenesis. Mitochondrial “mass” was measured by fluorescence microscopy after staining the mitochondria with Mitotracker green (a) or Mitotracker red (b) to assess mitochondrial proliferation. (A)
Axenically growing cells at the exponential phase were harvested by centrifugation at 3,000 x g for 15 s, washed once in Lo-Flo HL5 (3.85 g/L glucose, 1.78 g/L protease peptone, 0.45 g/L yeast extract, 0.485 g/L KH₂PO₄ and 1.2 g/L Na₂HPO₄·12H₂O; filter sterile), incubated in Lo-Flo medium for 2 h and then divided into two aliquots. One aliquot was resuspended in Lo-Flo HL-5 containing 200 nm Mitotracker Green FM (Invitrogen), whereas the other aliquot, resuspended in only Lo-Flo HL5 as an unstimulated control. Both aliquots were incubated for an hour in the dark and then unbound Mitotracker Green was removed by washing the cells three times in Lo-Flo HL5, with 10 min shaking on an orbital shaker (150 rpm) between washes. Finally, the cells were resuspended in Lo-Flo HL5 and fluorescence was measured using the Blue Module in a fluorometer (Turner Biosystems Modulus™).

The Mitotracker Green fluorescence per million cells was calculated after subtraction of the background fluorescence in the unstimulated cells. (B) Cells grown to log phase in HL5 medium were placed on sterile coverslips in six-well plates (Nalge Nunc™, Naperville, IL), washed gently in Lo-Flo HL5 and stained with 200 nm Mitotracker Red CMX-Ros (Invitrogen™) in Lo-Flo HL5 for 1 h in the dark. Unbound Mitotracker Red was removed by washing the cells 3 to 4 times in Lo-Flo HL5 over 2 h. After two washes in phosphate buffer (12 mm Na₂HPO₄, 12 mm NaH₂PO₄, pH 6.5), the cells were fixed and flattened at the same time by placing the coverslips up side down on a layer of 1% agarose in phosphate buffer containing 3.7% paraformaldehyde for 30 min. The fixed cells on the coverslips were washed four times (5 min each) in phosphate-buffered saline and mounted for microscopy. Images of cells and mitochondrial concentrations were obtained and analysed by fluorescence microscopy.

**ATP assays**

The ATP levels in cells of the AMPKα₃80 overexpressing and AMPK antisense-inhibited cells were measured. ATP assays were conducted using the luciferase-based ATP Determination Kit (Biaffin GmbH, Germany) in cells grown axenically in HL5 medium. Background luminescences measured before the assay were subtracted, and ATP concentrations were determined from a standard curve constructed using 10-fold serial dilutions of the ATP standard (1 x 10⁻⁷ M to 1 x 10⁻¹¹ M) in assay buffer.

**RESULTS**

The AMPK α subunit in Dictyostelium is encoded by a 2.6 kb gene annotated as Q9XY6 (www.expasy.org), AF118151 (www.ncbi.nlm.nih.gov/entrez) and DDB0215396 (http://www.dictybase.org/blast); comprised of five exons and four introns. The N-terminus holds a highly conserved catalytic core, S_TKc domain and identical APE and DFG motifs with other eukaryotic cells (Figure 1). It also has asparagine rich C-terminus.

**Plasmid copies and RNA expression**

The form of the AMPK α subunit that was overexpressed contained the entire catalytic domain but was truncated within the putative region responsible for autoinhibition and binding to the β subunit. The truncation of the AMPK α subunit in the catalytic domain created a constitutively active form of AMPK. Southern blot analysis for the strains overexpressing truncated AMPK α subunit and strains in which AMPK is antisense inhibited yielded bands that varied in their intensities (Figures 2A and 3A). This indicates different plasmid copies of the respective strains. The least prominent band showed intensity higher than AX2, the wild type strain (Figures 2 and 3). The strains exhibited a characteristic, stable level of RNA expression and the differences reflected the expression patterns in the genome (Figures 2B and 3B). Both plasmid constructs affected expression in a copy number-dependent manner. A steady state level of AMPKα₃80 mRNA in quantitative northern blots tightly correlated with the plasmid copies of the AMPKα₃80 expression construct insertions in the strain genomes (Figures 2A).

Overexpression mutants showed strong RNA expression signals of increasing intensities as a number of AMPK α₃80 construct increased (Figure 2). The sense RNA (control) also showed high RNA expression that tightly correlated with the plasmid copies (Figure 3B). By comparison with the sense RNA controls, the equivalent antisense RNA levels were dramatically reduced even at the lowest plasmid copies of the antisense plasmid construct (Figure 3). Interestingly, the extent of RNA reduction correlated with the plasmid copies of the antisense RNA-expression construct (Figure 3). This indicates degradation in the antisense RNA expressing transformants of both the antisense and the native mRNA. This would mean lower expression levels of the native mRNA. Accordingly, the plasmid copies of the corresponding constructs were used as AMPK α subunit expression index. To facilitate analysis and presentation of the data, positive values were assigned to plasmid copies for overexpression constructs while negative values for the AMPK α antisense construct.

**Mitochondrial mass**

Overexpression of the AMPK α catalytic domain resulted in a strong Mitotracker Green fluorescence per cell, whereas AMPK α antisense inhibition exhibited slight reduction of the fluorescence signal compared to that of AX2 cells (Figure 4). Additionally, there was 2 to 3 fold increase in mitochondrial mass in strains overexpressing AMPK α subunit compared to AX2. Examination of Mitotracker Red stain showed increased mitochondrial fluorescence in AMPK α₃80 overexpression strains but decreased in AMPK α subunit antisense strains compared to AX2 (Figure 5). Increased intensity of Mitotracker Red stain in AMPK α₃80 strains signify elevated mitochondrial mass per cell. The higher the plasmid copies in the constitutively active AMPK strain the more the fluorescence from the assayed cells (Figures 4 and 5). The increase in
Figure 1. (A). The plasmid map of genomic region encompassing AMPK α subunit (Chromosome 3 coordinates 589784–592434, Watson strand) (DictyBase http://dictybase.org) cloned into pZEF2. The AMPK α subunit in Dictyostelium is encoded by a 2.6k gene. (B). The AMPK α subunit amino acids sequences of Dictyostelium discoideum using CLUSTAL W sequence alignment program (http://expasy.org) showed 61% homology to mammalian cells (Homo sapiens and Rattus norvegicus), 56% to Drosophila melanogaster, 61% to Caenorhabditis elegans, 65% to Arabidopsis thaliana and 64% to Saccharomyces cerevisiae. The N-terminus contains a highly conserved catalytic core, S_TKc domain and identical APE (shaded blue) and DFG (shaded purple) motifs with other eukaryotic cells. The residue (tyrosine, Y) shaded pink is conserved and marks the start of the catalytic domain in all eukaryotes. The asterisk (*) means residues are identical in all sequences in the alignment. The colon (:) means conservative substitutions are present that is, residues are identical or replaced by amino acids with similar biochemical properties. The period (.) means partially conservative substitutions of similar amino acids. The residue asparagine (N) at position 166 is only present in D. discoideum. Residue numbers to the right of the figure. Each amino acid residue is represented with one letter code.
in mitochondrial mass is an indication of mitochondrial proliferation. In the same vein, the more severe the antisense inhibition, the more the apparent decrease in the Advanced Technology Program (ATP).

**Effects of AMPK α subunit expression on ATP levels**

Since mitochondrial biogenesis was stimulated in AMPKα380 overexpressing cells it is anticipated that ATP levels in these cells would be altered in a similar fashion. The ATP levels in AMPK α380 overexpression cells were significantly elevated in the range of 2 to 4 folds relative to the ATP levels in AX2 strain (Figure 6). The higher the plasmid copies, the more the ATP production per cell. These findings are consistent with the role of AMPK in energy homeostasis and infer that AMPK activation in response to chronic energy stress boosts ATP generation to maintain energy expensive cellular functions. Conversely, the severe the antisense inhibition, the more apparent the ATP decrease. Taken together, this finding provides genetic evidence that AMPK plays a critical role in mitochondrial proliferation. Thus, constitutive AMPK activation initiates signalling pathways that would result in mitochondrial biogenesis. These findings show that constitutive activation of AMPK cause increase in mitochondrial
mitochondrial content in *D. discoideum*. This constitutes direct evidence that genetic activation of AMPK culminates in mitochondrial biogenesis.

**DISCUSSION**

AMPK has been the focus of increasing attention for its fundamental roles in cellular energy homeostasis in healthy cells and in a variety of pathological situations, most notably diabetes, cancer (Hardie and Hawley, 2001; Winder and Hardie, 1999) and mitochondrial diseases (Bokko et al., 2007). Healthy cells AMPK, a heterotrimeric complex comprising of a catalytic α subunit (Crute et al., 1998), regulatory non-catalytic β (Thornton et al., 1998), and γ subunits (Carling et al., 1994), is activated very sensitively by stresses such as strenuous exercise, ischaemia or glucose deprivation (Salt et al., 1998). ATP hydrolysis yields ADP that helps replenish cellular ATP by donating a phosphate group to another ADP, forming
Figure 4. Effect of AMPK α subunit expression on mitochondrial mass in *Dictyostelium*. Mitochondrial "mass" was measured by fluorescence with the mitochondrion-specific dye, Mitotracker Green after subtraction of autofluorescence from unstained cells and presented as relative fluorescence units per $10^6$ cells. The blue circles with red borders represent individual strains each carrying the indicated number of copies of the AMPK α380 overexpression construct while gray circles stand for strains each carrying the indicated number of copies of AMPK α subunit antisense construct. Each red square represents a strain carrying both the chaperonin 60 antisense construct and the AMPK α antisense construct per genome. The dark blue circles represent the AX2, the wild type parental strain used as control. Compared to wild type cells, the Mitotracker Green fluorescence was higher in the case of AMPK α380 overexpression, but reduced in the AMPK α subunit antisense-inhibited strain cells. $R^2$ is the coefficient of variation and equals the square of the Pearson product-moment correlation coefficient. The significance probability is the probability of the observed results occurring under the null hypothesis that the correlation coefficient was zero. Negative values indicate the copy numbers of the AMPK α subunit antisense inhibition construct, while positive values indicate copy numbers of the overexpression construct.

an ATP and an AMP. The dramatic rise of AMP: ATP ratio leads to an activation of AMPK via allosteric activation (Adams et al., 2004; Scott et al., 2004) and phosphorylation by AMPK–kinase (AMPKK) (Weekes et al., 1994; Hawley et al., 1995; Stein et al., 2000) allowing ≥ 200-fold activity. Thus, AMPK functions as a metabolic masterswitch, or more accurately, ‘low fuel warning system’ in mammalian (Winder and Hardie, 1999; Hardie and Hawley, 2001) and other eukaryotic cells (Wilson et al., 1996; Carlson, 1999; Pan and Hardie, 2002). The activated cascade regulates metabolic pathways and promotes the adaptation of eukaryotic cells to their micro-environment in response to the ever changing energy charge (Hardie and Hawley, 2001; Suzuki et al., 2007). This, places AMPK holoenzyme at a central control point in maintaining cellular energy homeostasis. This pivotal role places AMPK in an ideal position to also play a wider role in regulating whole-body energy metabolism (Carling, 2004).

Given that all physiological processes depend on energy supply in the form of ATP, there are potentially many links with AMPK. Rapid progress in the field has also created great expectations that AMPK will be an important therapeutic target for the treatment of diabetes, obesity, cancer, cardiovascular disease and mitochondrial diseases. Irrespective of these outcomes,
**Figure 5.** Mitotracker Red fluorescence microscopy of representative AMPK overexpressing, AMPK antisense-inhibited strains and wild type cells. Vegetative cells from AX2, AMPKα$^{380}$ overexpressing and AMPK α antisense-inhibited strains were stained with the mitochondrion-specific dye, Mitotracker Red and analysed by fluorescence microscopy. Compared to wild type cells (panel A), the Mitotracker Red fluorescence was more intense in the cells from AMPKα$^{380}$ overexpression strains (panel B), but similar to the AMPK α antisense-inhibited cells (panel C).

we can be assured that AMPK research will provide many new insights into the integration of metabolism and physiological functions. Despite the complexities of energy metabolism, it is clear that the primary trigger for mitochondrial biogenesis is an inability of the mitochondria to produce energy in the form of ATP to match the corporal demand. Constitutive activation of AMPK approximates the persistent AMPK activation such as from stresses. The strains expressing AMPKα$^{380}$ had varied but stable plasmid copies of the plasmid construct and corresponding varied levels of RNA expression. The strains expressing the constitutively active Dictyostelium AMPK α$^{380}$ phenocopied the chronic activation of AMPK and exhibited increased expression of RNA manifolds the level in wild type cells.

Truncation of the mammalian AMPK α subunit catalytic domain created a constitutively active form of AMPK and expression was characterised by significant increase in activity of the AMPK (Crute et al., 1998). The constitutively active AMPK has also shown to be resistant to inactivation by protein phosphatase 2C (Woods et al., 2000). Strains expressing the antisense construct contained varied plasmid copies and corresponding RNA expression profile depicting diminution of RNA as the plasmid copies increased. Antisense inhibition of the AMPK α subunit led to significant reduction of expression and consequently attenuation of AMPK function. This indicates degradation in the antisense RNA expressing transformants of both the antisense and the native mRNA. This would mean lower expression levels of the native mRNA. During successful antisense, RNA inhibition, both strands of the mRNA and antisense RNA duplex, are degraded by the RISC complex (Hinas and Söderbom, 2007). Earlier attempts at creating a knockout strain or antisense inhibited strains with plasmid copies higher than 400, which were not successful. This possibly shows that strains in which AMPK is knocked out may not be viable. Additionally, strains with severely attenuated RNA expression beyond a particular threshold may also not survive. Moreover, strains with higher plasmid copies...
of antisense plasmids showed severe growth and development defects (Bokko et al., 2007).

Overexpression of a constitutively active form of the AMPK α subunit resulted in increased mitochondrial content, mitochondrial mass and ATP levels. The mitochondrial dyes (Mitotracker Red and Mitotracker Green) showed that AMPK α overexpression resulted in increased mitochondrial mass in Dictyostelium cells indicative of mitochondrial proliferation. By constitutive activation of AMPK, mitochondrial dysfunction can be countervened to generate energy. As in human cells, AMPK in Dictyostelium stimulates the proliferation of mitochondria and elevated ATP production. This finding corroborates Williams et al. (1986) and Zong et al. (2002) who reported that in mammalian cells, particularly in muscle tissues, AMPK activity leads to mitochondrial proliferation. Indeed, Zong et al. (2002) stated that AMPK is the proximal signalling step in stimulation of mitochondrial biogenesis in mammalian cells. This is part of the response to strenuous physical training in athletes and is a component of AMPK’s roles in energy homeostasis in healthy cells. Prolonged AMPK activation essentially sequel to mitochondrial dysfunction initiates mitochondrial biogenesis to buffer energy deficits and low ATP level in the cell in response to chronic energy deprivation. Thus, by sensing the energy status of the cell, AMPK initiates events culminating in mitochondrial biogenesis. Genetic activation of AMPK mimics activation, culminating in mitochondrial proliferation. Furthermore, the ATP levels were elevated in AMPK α subunit overexpression strains sequel to mitochondrial proliferation. This work adds to the multiple machinery channels that concertedly culminate in mitochondrial biogenesis.

Conversely, antisense inhibition of AMPK α subunit did not significantly affect mitochondrial content or ATP levels at lower plasmid copies. The ATP levels and mitochondrial content were similar to the wild type strain. As the functional attenuation by antisense inhibition becomes...
severe, the mitochondrial content and ATP level diminution becomes apparent. For instance, strain HPF461 with the highest plasmid copies, had the least fluorescence when stained with mitotracker green as well as lowest ATP level. Antisense strains may establish new energy threshold in the strains or had inapparent effect on AMPK activity with respect to mitochondrial content. Earlier reports suggested that mitochondria may undergo fusion with other mitochondria or become inactive when the energy use is low (Hermann et al., 1998; Chan, 2006). Furthermore, Jones et al. (2012) advocated that mitochondria alter their function according to prevailing cellular energetic requirements and thus function as sensors that generate signals to adjust fundamental cellular processes through a retrograde mitochondria-nucleus signalling pathway.

Conclusion

Taken together, this work show that AMPK plays a critical role in mitochondrial proliferation. Constitutive activation of AMPK initiates signalling to downstream pathways that culminates in increased mitochondrial mass in D. discoideum. This constitutes direct evidence that genetic activation of AMPK propels in vivo mitochondrial biogenesis and ATP generation in D. discoideum.

ACKNOWLEDGEMENTS

We gratefully acknowledge the support of La Trobe University Postgraduate Research Scholarship (LTUPRS) and the Australian Government International Postgraduate Research Scholarship Scheme (IPRSS) without which the present study could not have been completed.

Conflict of interest

Authors declare that there are no conflicts of interests

REFERENCES

Journal of Cell Biology and Genetics

Related Journals Published by Academic Journals

- Journal of Medicinal Plant Research
- African Journal of Pharmacy and Pharmacology
- Journal of Dentistry and Oral Hygiene
- International Journal of Nursing and Midwifery
- Journal of Parasitology and Vector Biology
- Journal of Pharmacognosy and Phytotherapy
- Journal of Toxicology and Environmental Health Sciences