Disruption of inositol biosynthesis through targeted mutagenesis in *Dictyostelium discoideum*: generation and characterization of inositol-auxotrophic mutants

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myo-Inositol and its downstream metabolites participate in diverse physiological processes. Nevertheless, considering their variety, it is likely that additional roles are yet to be uncovered. Biosynthesis of *myo*-inositol takes place via an evolutionarily conserved metabolic pathway and is strictly dependent on inositol-3-phosphate synthase (EC 5.5.1.4). Genetic manipulation of this enzyme will disrupt the cellular inositol supply. Two methods, based on gene deletion and antisense strategy, were used to generate mutants of the cellular slime mould *Dictyostelium discoideum*. These mutants are inositol-auxotrophic and show phenotypic changes under inositol starvation. One remarkable attribute is their inability to live by phagocytosis of bacteria, which is the exclusive nutrient source in their natural environment. Cultivated on fluid medium, the mutants lose their viability when de-

prived of inositol for longer than 24 h. Here, we report a study of the alterations in the first 24 h in cellular inositol, inositol phosphate and phosphoinositide concentrations, whereby a rapidly accumulating phosphorylated compound was detected. After its identification as 2,3-BPG (2,3-bisphosphoglycerate), evidence could be found that the internal disturbances of inositol homeostasis trigger the accumulation. In a first attempt to characterize this as a physiologically relevant response, the efficient *in vitro* inhibition of a *D. discoideum* inositol-polyphosphate 5-phosphatase (EC 3.1.3.56) by 2,3-BPG is presented.

Key words: 2,3-bisphosphoglycerate, *Dictyostelium*, inositol-3-phosphate synthase, gene disruption, inositol auxotrophy, phosphoinositide.

INTRODUCTION

Throughout the biological kingdom, the cyclitol *myo*-inositol and its metabolites are involved in diverse physiological processes such as growth regulation, membrane biogenesis and osmotolerance, apart from their significance in eukaryotic signal transduction. The biosynthesis of *myo*-inositol follows an evolutionarily conserved pathway [1]. All *myo*-inositol-producing organisms studied to date generate the cyclitol from Glc6P (glucose 6-phosphate) via an internal oxidoreduction and aldol cyclization reaction catalysed by MIPS (inositol-3-phosphate synthase; formerly known as L-*myo*-inositol-1-phosphate synthase; EC 5.5.1.4) in an NAD⁺-dependent manner [2]. Subsequent dephosphorylation of the product leads to unsubstituted inositol. The MIPS enzyme has been reported from a host of diverse sources (e.g. higher plants, animals, green algae, fungi, bacteria and archaea) and has been considered to be an ancient protein/gene [1]. The structural gene for MIPS, termed *ino1*, was first identified and cloned in *Saccharomyces cerevisiae* and has meanwhile been sequenced from evolutionarily diverse organisms. Deduced amino acid sequences reveal that eukaryotic MIPS are remarkably conserved throughout their length (≥45% identity) and that the following stretches of amino acid residues are highly conserved: GWGGNNG, LWTANTERY, NGSPQNTFVPGL and SYNHLGNNDG. The GXGGXXG motif (Rossman fold) is involved in NAD⁺ binding and is typical for an oxidoreductase.

The reaction catalysed by MIPS is the committed and rate-limiting step in the synthesis of all inositol-containing com-

pounds, making it an ideal target for genetic manipulation in our ongoing studies of the metabolism and functions of inositol phosphates/phosphoinositides in *Dictyostelium discoideum*. The typical characteristics of this cellular slime mould prompted the NIH to select it as a model organism for functional analysis of sequenced genes (http://www.nih.gov/science/models/d_discoideum/). *D. discoideum* has a complex life cycle with independent vegetative growth and multicellular developmental phases. Indeed this organism has unique advantages for the study of fundamental cellular processes resembling those in higher eukaryotes, which are absent or less accessible from other protists, such as yeast [3]. Examples in the unicellular organism are cell motility coupled with alterations in the actin/myosin-cytoskeleton, membrane trafficking especially associated with endocytosis, and signal transduction, as well as developmental aspects after initiation of differentiation, processes in which inositol derivatives such as inositol phosphates and phosphoinositides are most likely to be involved [4,5].

myo-Inositol is not an essential ingredient of well-defined culture medium [6], so cells are able to synthesize the compound *de novo*. Evidence given below supports the involvement of MIPS. Tritiated *myo*-inositol transferred into the cytoplasm by electroporation is incorporated into PtdIns within minutes [7]. Phosphorylation of PtdIns is accomplished by a family of five specific kinases [PIK1 (phosphoinositide kinase 1)–PIK5], whose corresponding genes have been cloned [8]. Site-directed mutagenesis provokes defects in the organization of the cytoskeleton and perturbations in differentiation. Certain double knockouts appear to

Abbreviations used: 2,3-BPG, 2,3-bisphosphoglycerate; CHO cell, Chinese-hamster ovary cell; DAG, diacylglycerol; *Dd*, *Dictyostelium discoideum*; DIG, digoxigenin; Fru(1,6)P₂, fructose 1,6-bisphosphate; Glc6P, glucose 6-phosphate; HPIC-CD, high-performance ion chromatography with conductivity detection; HPIC-IPAD, high-performance ion chromatography with integrated pulsed amperometric detection; MDD, metal dye detection; MIPS, inositol-3-phosphate synthase; PAR, 4-(2-pyridylazo)resorcinol; PIK1, phosphoinositide kinase 1; PI3K, phosphoinositide 3-kinase.

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be lethal. The phosphoinositide signalling cascade is involved in the processing of chemotactic stimuli (e.g. cAMP and folate). Stimulation of phospholipase C induces the formation of the second messengers DAG (diacylglycerol) and $\text{Ins}(1,4,5)\text{P}_3$ within seconds [7]. An inositol-polyphosphate 5-phosphatase participates in signal termination. Recently, a group of four inositol-polyphosphate 5-phosphatases from *D. discoideum* was cloned and characterized [9]. Hints about their substrate specificity were derived from overexpressed catalytic domains in *Escherichia coli*, and their possible physiological functions were implicated in the regulation of the signalling molecules $\text{PtdIns}(4,5)\text{P}_2$, $\text{PtdIns}(3,4,5)\text{P}_3$, $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$. Further inositol phosphate metabolism seems to be very complex, with approx. 25 identified compounds in this organism and very little information about the enzymes involved [7]. Remarkable are the relatively large concentrations of InsP_6 (~0.3–0.6 mM) in the exponential growth phase while being cultured either on bacteria or in axenic medium. InsP_6 and the compounds containing energy-rich pyrophosphate groups, 6-*PP*- InsP_5 (InsP_7) and 5,6-bis-*PP*- InsP_4 (InsP_8), are accumulated in stationary cells and in spores, where each of these reaches nearly millimolar concentrations [10]. In line with such observations, it may be speculated that these compounds may fulfil storage functions, serving as a unimolecular source of inositol, phosphate and also metal ions, because of their complexation properties, so that they correspond to the role of phytic acid (mixed calcium/magnesium salt of InsP_6) in plant seeds. This should certainly be important for ancient organisms like amoebae, with a resistant stage in their life cycle.

A co-ordinated inositol phosphate/phosphoinositide metabolism depends on the supply of inositol. Our efforts to manipulate the central biosynthetic pathway by insertional mutagenesis combined with an extensive analysis of phenotypic and metabolic changes in the mutants generated should yield new information about the biological importance of some inositol derivatives and their metabolic interrelationship. For instance practically nothing is known about possible interconnections between the metabolism of the signalling molecules at low concentrations, and the highly concentrated InsP_6 , InsP_7 and InsP_8 .

EXPERIMENTAL

Materials

All chemicals, buffers and medium constituents used were reagent grade or better and were purchased from Sigma, Roth or Oxoid. Enzymes were obtained from Sigma, Roche or New England Bioscience. *myo*-[^3H]inositol, Hybond-N+ nylon membrane, Superdex 200 HR, Mono Q and Source 15Q anion exchange material were from Amersham Biosciences. PAR [4-(2-pyridylazo)resorcinol] was obtained from Fluka and YCl_3 from Aldrich. Kits for purification of plasmids, DNA and RNA were obtained from Qiagen. The cDNA clone FC-AA11 was obtained from the Tsukuba cDNA bank. $\text{Ins}(1,4,5)\text{P}_3$ in cellular extracts was determined by the [^3H] $\text{Ins}(1,4,5)\text{P}_3$ Biotrak Assay System (no. TRK1000; Amersham Biosciences) according to the manufacturer's recommendations. The reference substance $\text{Ins}(1,4,5)\text{P}_3$, also used for the inhibition studies, was purchased as sodium salt from Sigma. All other phosphoinositides were obtained from Echelon.

Growth and development of *D. discoideum*

D. discoideum wild-type cells (strain AX2, A.T.C.C. 24397) and the mutant strains described were grown axenically in synthetic FM medium [6] and semi-synthetic HL5 medium [11] with shak-

ing (120 rev./min, 22 °C) or submersed in a tissue culture flask, supplemented with 10 µg/ml blasticidin S or G418 if necessary.

D. discoideum were also cultured in suspensions of *E. coli* B/r [10^{10} cells/ml in 40 mM phosphate buffer (containing potassium phosphate), pH 6.8; 120 rev./min, 22 °C] or in association with bacteria on SM agar plates [12]. Cells were harvested by centrifugation (500 g, 5 min and 4 °C). The cell density and the cell size of *D. discoideum* were determined with a Coulter® counter (model Z2). Chemotaxis was analysed by the agar-well assay [13]. Differentiation was initiated by plating washed, exponentially growing cells on non-nutrient agar plates (15 g/l agar in 40 mM phosphate buffer, pH 6.8).

Construction of the gene-disruption plasmid

The blasticidin-resistance cassette was excised from pUCBsrΔBam [14] by EcoRI and HindIII and treated with T4-DNA polymerase to yield blunt ends. The fragment was inserted into a single AspI site (position 568 of the longest open reading frame) of the cDNA of FC-AA11, which was also blunted before insertion. The resulting plasmid was termed pUCBsr-INO1. Before transformation, the plasmid was linearized with BamHI, which increased homologous recombination efficiency.

Antisense plasmid construction

A 1.5 kb BamHI/SalI fragment from FC-AA11 was inserted into pUC18 cleaved with BamHI and SalI. The 1.5 kb EcoRI/HindIII fragment from the resulting vector was cloned in antisense orientation in the EcoRI and HindIII sites that lie downstream of the *actin15* promoter in the *D. discoideum* expression vector pDEXRH [15], which also carries a G418-resistance cassette. The resulting plasmid was termed pDEX-INO1.

Transformation

Approximately 10^7 cells from the exponential growth phase were washed twice at 4 °C in electroporation buffer (21 mM Hepes, pH 7.1, 135 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4 and 5 mM glucose). After centrifugation (500 g, 5 min and 4 °C), the cell pellet was resuspended in 800 µl of ice-cold electroporation buffer. DNA (50 µg) was added and the suspension was transferred into a 4 mm electroporation cuvette. Electroporation was performed with a single pulse (1.25 kV, 25 µF; time constant: 0.5 ms) from the Gene Pulser (Bio-Rad).

Aliquots of the cell suspension (equal to $1\text{--}5 \times 10^6$ cells) were added to 10 ml of HL5 containing 500 µM *myo*-inositol and transferred to 80 mm Petri dishes. After 24 h, the medium was changed and the cells were kept in HL5 with 500 µM *myo*-inositol and 10 µg/ml blasticidin S (pUCBsr-INO1) or G418 (pDEX-INO1) for 8 days with a change of medium every 2–3 days. Potential mutants were screened by Northern blots and by enzymatic assays. Additionally, their growth in the presence or absence of *myo*-inositol was examined.

Isolation of RNA and Northern-blot analysis

Total RNA was extracted from *D. discoideum* cultures with the Qiagen RNeasy Midi/Mini kit according to the 'Protocol for Isolation of Cytoplasmic RNA from Animal Cells' (Qiagen RNeasy Midi/Maxi Handbook, Second edition 06/2001). RNA (1.5 µg) was size-fractionated on a formaldehyde-containing agarose gel, transferred on to a nylon membrane and probed with DIG (digoxigenin)-UTP-labelled RNA (DIG RNA Labelling kit; Roche). Hybridized RNA species were detected with a DIG luminescent detection kit (Roche).

Assay for MIPS

The protein preparation (20 μ l) was added to 80 μ l of assay mixture (62.5 mM Mops, pH 8.0, 20 mM NH_4Cl , 6.25 mM Glc6P and 1.25 mM NAD^+) and incubated for 1 h at 37 °C. The reaction was terminated by heating at 95 °C for 2 min, and precipitated proteins were removed by centrifugation (13 000 g, 2 min and 20 °C). Ins3P in the assay mixture was dephosphorylated by treatment with 5 μ l of reaction buffer (50 mM Mops, pH 9.0, and 80 mM MgCl_2) and 4 units of alkaline phosphatase (Roche) for 1 h at 37 °C. The reaction was stopped by heating at 95 °C for 2 min. The samples were then diluted to 2.5 ml with deionized water and subjected to HPIC-IPAD (high-performance ion chromatography with integrated pulsed amperometric detection).

Cell viability

Cell viability was determined by inoculating 4 ml of HL5 medium supplemented with 500 μ M *myo*-inositol in a tissue culture flask (surface area: 25 cm²) with approx. 100 cells. Each viable cell led to a visible clone within 5–6 days. Other methods, such as staining with a vital dye (problem: passive incorporation into dead cells) or counting of plaques on bacterial lawns (problem: mutants were unable to grow on bacteria), were inapplicable.

Endocytosis

Endocytosis was determined as described in [16]. Pinocytosis was assayed as fluorescence internalized by *D. discoideum* cells using FITC-labelled dextran (molecular mass 40 kDa) as a fluid phase marker. Phagocytosis was measured as fluorescence incorporated by *D. discoideum* cells through uptake of FITC-labelled *E. coli* B/t.

Analysis of inositol (HPIC-IPAD)

myo-Inositol from different sources, either accumulating in the MIPS assay or extracted from cells [uncharged fraction after treatment with Amberlite® MB-3 (Merck)] was determined by HPIC-IPAD as described in detail elsewhere [17]. The following gradient of NaOH was applied (0 min, 0 mM NaOH; 30 min, 250 mM NaOH; 40 min, 250 mM NaOH; flow rate 0.4 ml/min; see also Supplementary data at <http://www.BiochemJ.org/bj/397/bj3970509add.htm>). Excess glucose was eluted in a regeneration step with 1 M NaOH.

Analysis of Ins3P, 2,3-BPG (2,3-bisphosphoglycerate) and glycolysis metabolites [HPIC-CD (HPIC with conductivity detection)]

An HClO_4 extract of 5×10^8 cells was diluted to a conductivity of 3 mS/cm and applied to a Source 15Q HR 5/5 column. The column was washed with 10 mM HCl, and the glycolysis metabolites along with inositol mono- and bis-phosphates were eluted with 200 mM HCl. The resulting fraction was freeze-dried, diluted with water and subjected to HPIC-CD. The analysis was performed with a DX-500 ion chromatography system (DIONEX®) consisting of a gradient pump (GP50), a chromatography oven (LC30) with an internal valve bearing a 100 μ l sample loop, an absorbance detector (AD25) and an electrochemical detector (ED50) with a AMMS III suppressor. At a constant temperature of 30 °C, an IonPac AG11-HC (4 mm $\times$ 50 mm; DIONEX®) precolumn and an IonPac AS11-HC (4 mm $\times$ 250 mm; DIONEX®) analytical column were used. To separate the inositol monophosphates, the column was eluted isocratically with 10.5 mM KOH [water/methanol, 87.5:12.5

(v/v); flow rate 1.1 ml/min]. Ins3P had a retention time of 11.4 min.

The metabolite 2,3-BPG was purified by HPLC on Mono Q (details described in the Analysis of inositol phosphates and ATP subsection) from an HClO_4 extract of 10^9 *Ddino1* Δ 1 cells (where *Dd* is *Dictyostelium discoideum*) pre-incubated for 24 h on a medium without inositol. After freeze-drying, this method yielded 50–75 nmol of 2,3-BPG. The homogeneity of the compound was confirmed as described below. 2,3-BPG (30 nmol) was subjected to a 4 h enzymatic degradation with 250 m-units of phosphoglycerate mutase (EC 5.4.2.1; Sigma). The reaction was carried out at room temperature (20 °C) in 1.5 ml of 75 mM Bis-Tris (pH 7.4), and in the presence of 1.7 mM 2-phosphoglycolate (Sigma). The enzyme works in the presence of phosphoglycolate as a 2-phosphatase. The reaction was stopped by boiling, the suspension was cleared by centrifugation, and aliquots were analysed by HPIC-CD (0 min, 0.2 mM KOH; 5 min, 0.2 mM KOH; 15 min, 15 mM KOH; 25 min, 40 mM KOH; 45 min, 55 mM KOH; 55 min, 70 mM KOH; 70 min, 90 mM KOH; flow rate 1 ml/min). This gradient was also applied to separate and quantify other glycolysis metabolites from cellular extracts (3-phosphoglycerate, 25.2 min; phosphoglycolate, 25.7 min; Fru(1,6) P_2 (fructose 1,6-bisphosphate), 31.7 min; 2,3-BPG, 37 min).

Analysis of phosphoinositides (HPIC-CD)

Phosphoinositides were extracted with 9.5 ml of chloroform/methanol/12 M HCl/water (3:4:0.5:1, by vol.) from 2×10^9 cells. The mixture was incubated at room temperature (30 min, ultrasonic bath) and 3.75 ml of chloroform and 3.75 ml of water were subsequently added. After vortex-mixing and centrifugation (5000 g, 10 min and 20 °C), the lower organic phase was carefully transferred to another tube. The aqueous phase was discarded, and the interphase was extracted with 3.75 ml of chloroform. The combined organic phases were evaporated to dryness on a rotary evaporator.

The lipid samples were deacylated by the method described previously [18]. Prior to HPIC-CD, hydrophobic contaminations were removed by an RP/H Maxi-Clean cartridge (Alltech). Aliquots of 2×10^7 cells were used for analysis. To elute glycerophosphoinositol and the glycerophosphoinositol phosphates, we used a water/methanol mixture (75:25, v/v) with a KOH gradient (0 min, 5 mM KOH; 5 min, 5 mM KOH; 15 min, 30 mM KOH; 25 min, 30 mM KOH; 35 min, 60 mM KOH; 45 min, 60 mM KOH; 50 min, 90 mM KOH; 60 min, 90 mM KOH; flow rate 0.85 ml/min). Compounds and their corresponding retention times: glycerophosphoinositol, 4.54 min; glycerophosphoinositol 4-phosphate, 23.54 min; glycerophosphoinositol 3-phosphate, 24.83 min; glycerophosphoinositol 4,5-bisphosphate, 42.95 min.

Deacylated PtdIns could not be quantified by HPIC-CD, because further substances co-eluted with it (one of these is probably deacylated phosphatidylglycerol [19]). To determine cellular PtdIns concentrations, the deacylation product glycerophosphoinositol was recovered after HPIC-CD and converted in two steps into *myo*-inositol.

Freeze-dried samples derived from 5×10^7 cells were dissolved in 400 μ l of water, and 50 μ l of 90 mM NaIO_4 was added to oxidize the glycerol backbone (20 min, room temperature, darkness). Excess NaIO_4 was destroyed by addition of 20 μ l of 700 mM Na_2SO_3 (20 min, room temperature, darkness). Dimethylhydrazine [40 μ l; 1% (w/v); reagent was adjusted to pH 4.5 with formic acid prior to use] was added, and the reaction mixture was incubated for 4 h to convert the resulting glycolaldehyde into Ins1P. Ins1P was dephosphorylated by treatment with 1 ml of 50 mM Mops (pH 9.0) and 10 units of alkaline phosphatase

for 4 h at 37 °C. The reaction was stopped by heating at 95 °C for 2 min. The samples were deionized on a mixed bed ion exchanger (Amberlite® MB-3; Merck), and inositol was quantified by HPIC-IPAD.

Analysis of inositol phosphates [HPLC–MDD (metal dye detection)] and ATP

HClO₄ extracts were prepared as described previously [10]. The inositol phosphates were separated by anion-exchange chromatography on a Mono Q HR 10/10 column with a linear gradient of HCl (0 min, 0.2 mM HCl; 70 min, 0.5 M HCl; 90 min, 0.5 M HCl; flow rate 1.5 ml/min). InsP₆–InsP₈ were separated with the following gradient (0 min, 0.2 M HCl; 15 min, 0.5 M HCl; 25 min, 0.5 M HCl; flow rate 1.5 ml/min) on a Source 15Q HR 5/5 column. Photometric detection at 546 nm was achieved by post-column derivatization with a metal–dye reagent {2 M Tris/HCl, pH 9.1, 200 µM PAR, 30 µM YCl₃ and 10 % (v/v) methanol; flow rate 0.75 ml/min; [20]}.

ATP was determined after application of the samples to a Source 15Q HR 5/5 column, which was eluted by a linear gradient of HCl (0 min, 0.2 mM HCl; 15 min, 0.5 M HCl; 20 min, 0.5 M HCl; flow rate 1.5 ml/min). UV detection was performed at 254 nm (ATP, 13.5 min).

Partial purification of an inositol-polyphosphate 5-phosphatase (*Dd5P4*), inhibition studies

The corresponding 5-phosphatase from *D. discoideum* strain AX2 was enriched from cytosolic extracts by chromatography on heparin–agarose (Sigma) as described in [21]. A 5 mg portion of the concentrated 100 mM NaCl fraction (Centriprep® YM-50; Millipore) was subjected to size-exclusion chromatography on a Superdex 200 HR 10/30 column (Amersham Biosciences; fraction size 0.3 ml; flow rate 0.3 ml/min). The column was eluted with 40 mM Tris/HCl (pH 7.7), 50 mM NaCl and 1 mM EDTA. The fractions were analysed for 5-phosphatase activity, and the active fractions eluting in the range of 80–120 kDa were pooled (specific activity ~50 m-units/mg) and utilized for the inhibition studies {molecular mass of the enzyme purified to homogeneity 95 kDa (native) and 92 kDa (SDS/PAGE) [22]}.

Activity was determined by a modification of the method of Van Lookeren Campagne et al. [23]. The assay was carried out on a microplate in a solution composed of the following components (per cavity): 10 µl of buffer (160 mM Bis-Tris, pH 7.0, 800 mM sucrose, 1 mM EDTA and 20 mM MgCl₂), 10 µl of water or a solution of the inhibitor 2,3-BPG (0.03–2 mM), 10 µl of the corresponding protein fraction and 10 µl of 0.64 mM Ins(1,4,5)P₃ solution. For further details, please consult one of our earlier publications [21].

RESULTS AND DISCUSSION

Gene replacement and antisense-mediated gene inactivation

The *D. discoideum* cDNA database (<http://dictydb.biol.tsukuba.ac.jp/>) and the *D. discoideum* genomic database (<http://dictybase.org/>) were screened for putative MIPS homologues containing the conserved motifs (see the Introduction section). A cDNA (FC-AA11) of approx. 1.5 kb was found. The deduced amino acid sequence showed identities between 45 and 60 % to those of MIPS from other organisms. The corresponding gene (*ino1*, DDB0231710) is located on chromosome 4 with a full length of 1672 bp, including one intron at position 1389–1528. The gene codes for a protein of 511 amino acids.

Figure 1 Molecular defects of the mutants *Ddino1Δ1* and *Ddino1as1* as demonstrated by MIPS activity assays and Northern-blot analysis

(A) A blot containing 1.5 µg of RNA in each lane isolated from vegetative cells cultured on FM medium in the presence of 500 µM *myo*-inositol (+) or 24 h without *myo*-inositol (–) was probed with DIG-UTP-labelled complementary RNA. (B) MIPS activity comparison between the mutants and AX2. The results are presented as the means ± S.D. for three independent experiments.

A stringent requirement for generating vital inositol-auxotrophic mutants is the uptake of external *myo*-inositol that will bypass the deficiency in inositol biosynthesis. Our experiments and previous studies [24] monitoring the incorporation of *myo*-[³H]inositol showed that this is possible for *D. discoideum* by pinocytosis. Gene disruption by homologous recombination requires additionally that only a single copy of the gene exists. Southern-blot analysis of genomic DNA digested with restriction enzymes not present in the cDNA sequence of FC-AA11 detected only one hybridizing fragment with a ³²P-labelled cDNA probe. It is not possible to get mutants by a gene knockout if functional *ino1* is essential for cell viability. In this case, it is preferable to choose the antisense strategy for gene silencing. For both strategies, vectors were constructed and expressed in *E. coli*. Transformation of AX2 cells results in three independent transformants by gene disruption (*Ddino1Δ1*–*Ddino1Δ3*) and 18 independent transformants by antisense mutagenesis (*Ddino1as1*–*Ddino1as18*). Two strains (*Ddino1Δ1* and *Ddino1as1*) showing the most pronounced effects on MIPS expression were selected for further studies. Their molecular defects were confirmed by enzyme activity assays and Northern blots (Figure 1).

The method applied to determine MIPS activity is based on the complete dephosphorylation of the reaction products and quantification of *myo*-inositol after HPIC-IPAD. As a further benefit, it allowed the partial purification and the preliminary characterization of MIPS from the parent strain AX2 (for details on HPIC-IPAD and the enzyme characteristics, see Supplementary data at <http://www.BiochemJ.org/bj/397/bj3970509add.htm>).

Phenotypic characterization of the mutants

Growth and differentiation

To prove a potential auxotrophy of the mutants, the cells were cultivated on a medium with no fluctuations in its composition [6]. On this synthetic medium, agitated cultures of the parent strain AX2 did not require exogenous *myo*-inositol for optimal growth, indicating that endogenous biosynthesis is adequate. In contrast, the mutants *Ddino1Δ1* and *Ddino1as1* are indeed inositol-auxotrophic and grow only after *myo*-inositol supplementation. At concentrations <100 µM *myo*-inositol, no growth of *Ddino1Δ1* is detectable (Figure 2). In the presence of 500 µM *myo*-inositol,

Figure 2 Growth of *Ddino1Δ1* and the parent strain AX2 at different *myo*-inositol concentrations

Cells were grown on FM medium with 500 μ M *myo*-inositol to a cell density of 2×10^6 cells/ml, washed with phosphate buffer, and used to inoculate FM medium containing *myo*-inositol as indicated (*Ddino1Δ1*: 0 μ M, $\blacklozenge$; 100 μ M, $\blacksquare$; 250 μ M, $\triangle$; and 500 μ M, $\square$; AX2: 0 μ M, $\blacktriangle$).

Figure 3 Morphological differences between *Ddino1Δ1* and the parent strain AX2

Photographs of vegetative cells (A) and two differentiation stages (B, C).

the growth rates of the mutants are similar to those of AX2 cells. Where not otherwise noted, the phenotypic and metabolic changes are demonstrated specifically for the knockout strain *Ddino1Δ1*. It has to be stated that the effects observed for the antisense mutant *Ddino1as1* are analogous, but are expressed less strongly. Transferred to *myo*-inositol-free medium, *Ddino1Δ1* cells stop growing completely, become smaller and lose their typical amoeboid form within 24 h (Figure 3A).

In the first 5–6 h of starvation (half the doubling time), the cells increase in number in parallel with the inositol-supplemented control, suggesting that their internalized reserves are sufficient. Then the cell number enters a plateau, but the cells remain viable for the next 18–20 h, as demonstrated by their ability to resume

Figure 4 Survival of *Ddino1Δ1* after sustained inositol deficiency

The results are presented as the means $\pm$ S.D. for three independent experiments.

growth after *myo*-inositol addition. Afterwards the cells lose their viability (Figure 4). Therefore the time period of 24 h seems to be optimal for observing pronounced effects of inositol starvation on the phenotype.

Inositol-auxotrophic cell lines from diverse organisms, including *S. cerevisiae* [25], *Neurospora crassa* [26] and mammalian cells [27], share a phenomenon called 'inositolless death'. Under conditions that otherwise support growth, the cells die when deprived of *myo*-inositol. Shatkin and Tatum [28] argued that 'inositolless death' is caused by an imbalance between the rate of membrane growth and the synthesis of cytoplasmic constituents. In agreement with their hypothesis, life span of *Ddino1Δ1* is prolonged when protein biosynthesis is blocked by cycloheximide or when they are additionally deprived of glucose, their main carbon source (results not shown).

In their natural environment, *D. discoideum* live on phagocytosis and digestion of bacteria. The parent strain can also be grown on agar plates in association with bacteria or in bacterial suspension. The mutants were unable to do so. This could be a consequence of the bacteria *E. coli* B/r and *Klebsiella aerogenes* we used, because these prokaryotes contain no *myo*-inositol. It seems puzzling that, in contrast with the growth defect observed on axenic medium, this was independent of *myo*-inositol supplementation, but it is known that pinocytosis rates of cells cultured on bacteria are almost negligible [29]. A deficient *myo*-inositol supply may cause a disturbed inositol phosphate/phosphoinositide turnover with potential impacts on vital processes, such as utilization of nutrients [30]. This aspect was studied further (see below).

Ddino1Δ1 showed only minor defects in chemotaxis and differentiation. In the small droplet chemotaxis assay, the migration of the cells towards cAMP on agar plates with or without *myo*-inositol was examined. Independently of the substrate chosen, gene inactivation does not affect chemotaxis (results not shown). In comparison with the parent strain plated on non-nutrient phosphate-buffered agar plates to initiate multicellular development, *Ddino1Δ1* forms greater multiple-tipped mounds (Figure 3B), developing into fruiting bodies with the typical morphology (Figure 3C). On an average, the mutants need 20% more time to differentiate. These defects cannot be suppressed by inositol supplementation. Mounds with multiple tips were also observed for a double knockout of two PI3Ks (phosphoinositide 3-kinases) (DdPIK1 and DdPIK2) closely related to mammalian p110 PI3K [8]. Another report describes the identification of a developmentally regulated PI4K that is highly active in post-aggregate stages [31]. Altogether, these results give hints of a relationship between phosphoinositide metabolism and multicellular development.

In the course of development, *D. discoideum* cells do not ingest nutrients from their environment; cells of *Ddino1Δ1* therefore

Figure 5 Fluid-phase uptake and phagocytosis of bacteria

(A) Comparison of fluid-phase uptake of AX2 (▲) with *Ddino1Δ1* in the presence of 500 μM *myo*-inositol (■) and after 24 h *myo*-inositol starvation (□). Pinocytosed volume is referred to surface area. *Ddino1Δ1* (–) cells needed a lag-phase of approx. 30 min to recover from the washing procedure. (B) Uptake of FITC-labelled *E. coli* B/r by AX2 (▲) and *Ddino1Δ1* at different times of *myo*-inositol starvation (0 h, ■; 8 h, ○; 16 h, △; 24 h, □).

have to access their internal reserves, especially for inositol. These resources seem to be sufficient to allow differentiation, even when the cells are inositol-starved for 24 h (middle trace of Figure 3).

Inoculated on medium without *myo*-inositol, spores are also capable of germinating, but the resulting cells die within the next few hours. In the presence of *myo*-inositol, however, the cells resume growth normally, reflecting once more their need of inositol for vegetative growth. It can be concluded that the continuous generation of at least one inositol-containing compound is essential for vegetative growth.

Pino- and phago-cytosis

Pinocytosis is the exclusive route for entry of nutrients into cells cultured on liquid medium [29]. Whether the growth defect of the mutants observed under inositol starvation correlates with alterations in pinocytosis was measured with the fluid-phase marker FITC-dextran. Pinocytosis rate and capacity are not influenced by *ino1* gene inactivation, even when the cells tested were inositol-starved for 24 h (Figure 5A). Fluid internalization seems not to be a growth-limiting factor.

The inositol-auxotrophic mutants are unable to grow on bacterial lawns or in suspensions of bacteria. Is this a consequence of a disturbed phagocytosis? In the presence of *myo*-inositol, phagocytosis of *Ddino1Δ1* cells is comparable with that of the parent strain. No significant difference was observed for the internalization of *E. coli* B/r, *K. aerogenes* or latex beads (Figure 5B, shown only for FITC-labelled *E. coli* B/r). At 16 h of *myo*-inositol starvation, the uptake rate of bacteria and the phagocytosis capacity of the cells are reduced. After 24 h *myo*-inositol starvation, the cells completely lose their ability to phagocytose (Figure 5B).

In contrast with pinocytosis under advanced inositol deficiency, the mutants are defective in the uptake of particles. The results suggest that the processes have different dependencies on inositol-containing compounds. Phagocytosis and subsequent digestion consist of a number of stages, including the binding of a particle to the cell surface, engulfment of the particle by pseudopod extension, and fission and fusion reactions to form phagolysosomes. It is well known that phosphoinositides are required for the internalization process in a number of cell types [32,33] and that they play a role in phagosomal maturation in *D. discoideum*

Table 1 Cellular concentrations of several inositol metabolites and 2,3-BPG in the strains AX2, *Ddino1Δ1* cultured on medium with 500 μM *myo*-inositol (+) and *Ddino1Δ1* after 24 h of *myo*-inositol starvation (–)

To calculate the concentrations, the following mean cell volumes were used: 751 $\pm$ 20 fl (AX2), 606 $\pm$ 19 fl [*Ddino1Δ1* (+)] and 513 $\pm$ 16 fl [*Ddino1Δ1* (–)]. Results are means $\pm$ S.D. for five independent experiments.

C_i (intracellular concentration; μM)	AX2 (–)	<i>Ddino1Δ1</i> (+)	<i>Ddino1Δ1</i> (–)
<i>myo</i> -inositol	45 $\pm$ 5	483 $\pm$ 175	3.8 $\pm$ 1.4
Ins3P	0.6 $\pm$ 0.2	1 $\pm$ 0.3	0.8 $\pm$ 0.2
2,3-BPG	6.1 $\pm$ 0.9	3 $\pm$ 0.7	135 $\pm$ 18
Ins(1,4,5) P_3	0.58 $\pm$ 0.11	0.80 $\pm$ 0.14	0.73 $\pm$ 0.14
Ins(1,2,3,4,6) P_5	15.2 $\pm$ 3.2	12.1 $\pm$ 3	22 $\pm$ 3
Ins(1,2,4,5,6) P_5	22.1 $\pm$ 3	18 $\pm$ 2	28.3 $\pm$ 4
Ins P_6	660 $\pm$ 35	513 $\pm$ 48	454 $\pm$ 45
Ins P_7	88 $\pm$ 17	76 $\pm$ 16	96 $\pm$ 21
Ins P_8	264 $\pm$ 29	375 $\pm$ 39	441 $\pm$ 39
PtdIns	64 $\pm$ 7	125 $\pm$ 15	5.4 $\pm$ 1.2
PtdIns4P	5.3 $\pm$ 0.4	4.1 $\pm$ 0.5	4.7 $\pm$ 0.3
PtdIns3P	3.5 $\pm$ 0.4	5 $\pm$ 0.9	3.8 $\pm$ 0.4
PtdIns(4,5) P_2	7.5 $\pm$ 0.9	6 $\pm$ 0.5	2.3 $\pm$ 0.3

[30]. This aspect will be taken up again when considering the results of the metabolite analyses.

Alterations in inositol-containing metabolites

Water-soluble compounds

Soluble inositol metabolites of *Ddino1Δ1* were analysed after 24 h of inositol starvation. At the beginning of the experiment, cellular levels of the common precursor inositol are usually high, varying between 350 and 600 μM , which seems to be the consequence of an accumulation of the incorporated cyclitol in endocytic compartments rather than uniform distribution in the cytosol. The basal level of the parent strain AX2, which needs no supplementation of *myo*-inositol to the fluid medium, lies in the range 40–60 μM . We assume that this steady-state level is optimal for vital functions in an exponentially growing culture. Keeping *Ddino1Δ1* cells for 24 h on inositol-free medium, only 3–4 μM are preserved (Table 1), confirming that MIPS indeed catalyses the committed and rate-limiting step in the *de novo* synthesis of *myo*-inositol.

The level of Ins3P, the actual product of the conversion of Glc6P by MIPS, was determined by HPLC-CD. It is maintained between 0.6 and 1.0 μM . It should be noted that an achiral method was used, and the enantiomer Ins1P could be present as well. Furthermore, other metabolic pathways might lead to the formation of Ins1P and Ins3P.

An isotope dilution assay was used to quantify Ins(1,4,5) P_3 . No significant alteration of the Ins(1,4,5) P_3 concentration (0.6–0.8 μM) occurs after inositol starvation.

As found for plant seeds, some highly phosphorylated inositol phosphates, particularly inositol hexakisphosphate, may fulfil storage functions and serve as a unimolecular source of *myo*-inositol, phosphate and also metal ions on account of their complexation properties [34]. Typical intracellular concentrations of Ins P_6 (up to 600 μM), Ins P_7 (up to 100 μM) and Ins P_8 (up to 300 μM) in *D. discoideum* [10] are high enough, so that complete dephosphorylation could compensate for a deficiency of intracellular *myo*-inositol over a longer period. HPLC-MDD analysis shows only minor fluctuations in the levels of inositol hexakisphosphate and the diphosphoinositol phosphates after inositol starvation (Table 1 and Figure 6), but Ins P_6 concentration

Figure 6 HPLC-MDD analysis of inositol phosphates from *Ddino1Δ1* extracts

(A) Separation of metabolites with up to five phosphate groups extracted from 2×10^8 *Ddino1Δ1* cells cultured on FM medium in the presence of $500 \mu\text{M}$ *myo*-inositol (+) or from an equal amount of cells kept for 24 h on FM medium without *myo*-inositol (–). (B) An analogous experiment with the extract from 1×10^7 cells and a gradient separating InsP_6 , InsP_7 and InsP_8 .

tends to decrease, whereas InsP_7 and InsP_8 concentrations increase. Taking into account that the mean cellular volume of the mutants is smaller, especially after *myo*-inositol starvation (see legend of Table 1), the apparent concentration of a metabolite that is not subject to variations will increase. A small proportion of the InsP_6 pool is actually decomposed, but the phenotypic changes and the inositol analyses indicate that this is not enough to compensate for the inositol demand.

Various explanations are conceivable for why such high concentrations of the highly phosphorylated inositol phosphates are conserved. A simple explanation may be that their metabolism is too slow to meet the needs for inositol in vegetative growth. From metabolic labelling studies of amoebae with [^3H]*myo*-inositol, it is known that the marker accumulates relatively slowly with the compounds InsP_6 , InsP_7 and InsP_8 [24]. However, some essential function could be fulfilled by those compounds later in development. As we have shown, differentiation is not fundamentally impaired even if *Ddino1Δ1* cells are inositol-starved for 24 h (Figure 3). In contrast, the cells die afterwards, when development is not initiated. Two recent publications describing the gene inactivation of the inositol phosphate kinases involved in the biosynthesis of highly phosphorylated inositol phosphates in mammals confirm the physiological relevance of all or some of these metabolites in development [35,36], and this may be also the case for *D. discoideum* [10]. However, deletion of the InsP_6 -kinase abolished the synthesis of InsP_7 and InsP_8 and resulted in a strain with accelerated aggregation but unimpaired differentiation [37], so it is unlikely that the pyrophosphorylated compounds are significantly involved. InsP_6 or the pentakisphosphates remain to be considered. We are currently studying an inositol pentakisphosphate 3/5-kinase [38] that participates in InsP_6 homeostasis, and hope to obtain further information about a possible function of such a kind soon by inactivating the corresponding gene.

Surprisingly, in the metabolism of inositol-starved mutants, there accumulates to a considerable extent a substance that is not a primary inositol metabolite (Figure 6A). On the basis of co-elution of the purified compound with authentic standards under several sets of HPLC conditions and a specific enzymatic degradation *in vitro* with concomitant identification of the products, we were able to identify it unequivocally as 2,3-BPG. Both *Ddino1Δ1* cells and those of the antisense mutant *Ddino1as1*, which were generated by a completely different method that targets *inol* expression, accumulate 2,3-BPG under inositol starvation (the

effect on cellular 2,3-BPG for *Ddino1as1* is approx. 1/3 of that described in Table 1). The potential physiological relevance of this observation is discussed later.

Phosphoinositides

Organic-solvent-extractable phosphoinositides were analysed after deacylation. Interestingly, *myo*-inositol supplementation to the medium enhanced the steady-state PtdIns level significantly [*Ddino1Δ1* and AX2 ($500 \mu\text{M}$ *myo*-inositol) ~ 100 – $125 \mu\text{M}$; AX2 (without supplementation) ~ 60 – $70 \mu\text{M}$], whereas the steady-state levels of all PtdInsPs investigated were unchanged by addition of *myo*-inositol. After 24 h starvation, the PtdIns level of *Ddino1Δ1* clearly decreased below the basal level to approx. $5 \mu\text{M}$ (Table 1). Phosphoinositide biosynthesis starts with the formation of PtdIns from *myo*-inositol and CDP-DAG catalysed by PtdIns synthase (EC 2.7.8.11), so it seems consistent that cellular inositol and PtdIns concentrations are both affected. A comparable influence of inositol starvation on PtdIns was reported for inositol-auxotrophic cells of *N. crassa* [26], *S. cerevisiae* [39] and CHO (Chinese-hamster ovary) cells [27]. PtdIns is one of the main components of membranes in *D. discoideum*, making up in vegetative cells 6% and in the plasma membrane 8% of all phospholipids [40]. It was found that in inositol-auxotrophic CHO cells the decreasing content of PtdIns was compensated for by phosphatidylglycerol [27], which helped to retain the cell dimensions. Despite additional two-dimensional TLC, we were unable to detect such a shift in phospholipid composition (results not shown). Accordingly, inositol-starved *Ddino1Δ1* cells became smaller, and it can be hypothesized that the disturbance in membrane biogenesis is associated with the early cessation of cytokinesis.

PtdIns is the exclusive precursor for all PtdInsPs that play a decisive role in cellular signalling. The concentration of the downstream metabolite PtdIns4P is nearly unaffected after inositol starvation (Table 1). This also holds for the PtdIns3P concentration. The level of the $\text{Ins}(1,4,5)\text{P}_3$ precursor PtdIns(4,5) P_2 decreases, but not so drastically as PtdIns. Unfortunately, the sensitivity of the HPLC system does not permit the direct determination of other biologically relevant PtdInsPs, especially of PtdIns(3,4,5) P_3 . It can be concluded that their concentrations are clearly below $0.2 \mu\text{M}$. In comparison with PtdIns, the intracellular levels of the PtdInsPs investigated are not as strongly affected. It seems that a mechanism exists that stabilizes the levels in response to internal disturbances of inositol homeostasis. In a genetically engineered strain with inositol biosynthesis destroyed, a compensation of this kind can be only temporary. Therefore, after 24 h, re-synthesis of PtdInsPs should only be possible to a very limited degree. Experiments with macrophages indicate that phagocytosis is accompanied by localized biphasic changes in PtdIns(4,5) P_2 concentration, which contribute to cytoskeletal remodelling [32]. The observed failure in phagocytosis after 24 h of inositol starvation (see Figure 5B) could be a consequence of an impaired PtdIns(4,5) P_2 metabolism. Thereafter the cells also lose their vitality (see Figure 4). Of course this may happen for several reasons, but one possible explanation could again be PtdIns(4,5) P_2 . Recent publications on the physiological functions of PtdIns(4,5) P_2 indicate that continuous synthesis of PtdIns(4,5) P_2 is essential to maintain the integrity of the Golgi apparatus [41]. Disintegration of this organelle will inevitably lead to cell exitus.

Drastic changes in the level of 2,3-BPG: potential cellular target

2,3-BPG is a product of a glycolysis branch called the 2,3-BPG shunt. Consuming 1,3-BPG the shunt operates with the

Figure 7 Kinetics of the concentrations of selected inositol metabolites and 2,3-BPG in the strain *Ddino1Δ1* subjected to inositol starvation

(A) Water-soluble inositol-containing compounds: Ins(1,4,5)P₃ (□), 2,3-BPG (●) and myo-inositol (▲). (B) PtdIns and PtdInsPs: PtdIns (■), PtdIns4P (○) and PtdIns(4,5)P₂ (△). Data are from one experiment, which is representative of at least three experiments.

expenditure of one equivalent ATP per triose passed through it. A disturbed glycolysis or energy metabolism could probably cause the 2,3-BPG changes observed under inositol deficiency. For that reason, we first examined the effects of inositol starvation on Fru(1,6)P₂ and ATP concentrations, to exclude a metabolic dysfunction that results secondarily in a 2,3-BPG increase. Neither the phosphofructokinase product nor ATP underlies significant fluctuations in the first 24 h [*Ddino1Δ1* (+) or *Ddino1Δ1* (–): Fru(1,6)P₂ = 254 ± 22 μM, ATP = 935 ± 96 μM comparable with results presented in [42]].

There is at least one ancient function of 2,3-BPG in glycolysis that makes it indispensable for all eukaryotic cells. To maintain the active form of phosphoglycerate mutase, catalytic amounts of 2,3-BPG are needed. *In vitro*, 2,3-BPG has often been used as a competitive inhibitor of inositol-polyphosphate 5-phosphatases in enzyme assays, and in the case of insulin-secreting pancreatic islets the physiological relevance of this inhibition was postulated [43]. There are indications of a reciprocal action: that inositol phosphates, especially Ins(1,4,5)P₃, interact with aldolase [44], and that the Ins(1,4,5)P₃ receptor associates with glyceraldehyde-3-phosphate dehydrogenase [45]. These literature results encouraged us to analyse the time course of the changes in cellular inositol, Ins(1,4,5)P₃ and phosphoinositide concentrations in relation to the 2,3-BPG concentration, as provoked by myo-inositol starvation in the first 24 h (Figures 7A and 7B). This might be an indicator of a causal relationship.

Ddino1Δ1 cells cultured on synthetic medium supplemented with 500 μM myo-inositol show artificially high cellular myo-inositol levels due to filled endocytic compartments. Thus, to get a realistic picture before the experiment was started, the cells were pre-incubated for 2 h, just the time needed to reach a new equilibrium between pinocytosis and exocytosis and when the myo-inositol levels were still slightly higher than the basal level of the parent strain (40–60 μM). The intracellular myo-inositol concentration of the mutant falls very far below this value in the first 2 h (Figure 7A). Afterwards it decreases only slightly, reaching a plateau of approx. 3–4 μM at 14 h of starvation. Directly opposed are the alterations in the glycolysis metabolite 2,3-BPG. Its concentration in the parent strain and *Ddino1Δ1* (+) varies between 2 and 3 μM. Coinciding with a deficit in cellular myo-inositol, after 2 h the level rises 4-fold and then continuously to a maximum at 14 h, when it remains at a level up to 50 times that of basal. In contrast, the cellular Ins(1,4,5)P₃ concentration fluctuates only within the error margins over the whole 22 h (0.6–0.8 μM nearly equal to [Ins(1,4,5)P₃]_i in AX2, see Table 1). On

Figure 8 Study on the inhibition of an inositol-polyphosphate 5-phosphatase (*Dd5P4*) by 2,3-BPG

Inorganic phosphate released from Ins(1,4,5)P₃ by an inositol-polyphosphate 5-phosphatase in the absence and presence of different concentrations of 2,3-BPG was determined and reaction rates were derived from the linear regions of kinetic data plots. Plotting the ratio of reaction rates of uninhibited and inhibited reaction against concentration of the inhibitor at constant initial substrate and enzyme concentrations results in a linear curve. The *K_i* value for 2,3-BPG was calculated from the slope, as described in [46].

starvation, the PtdIns level decreased below the basal value in the first 2 h, and the decline came to rest after approx. 14 h at a low level of approx. 6 μM (Figure 7B). Thus the time dependencies of the myo-inositol and the PtdIns concentrations show striking similarities in the progression and the minimal value finally reached, which is in both cases approx. 1/10 of the value found in the parent strain AX2. Despite the considerable loss of PtdIns, the concentration of the downstream metabolite PtdIns4P is subject to only minor variations over the 22 h examined. This also applies for the PtdIns3P concentration (results not shown). The level of the Ins(1,4,5)P₃ precursor PtdIns(4,5)P₂ decreases for 6 h to approx. 1/3 of the initial value with a slight tendency to recover afterwards. Of the metabolites that vary, the PtdIns(4,5)P₂ level normalizes fastest after a renewed myo-inositol supplementation. It needs only 4 h, followed by PtdIns and 2,3-BPG (~8 h; results not shown).

We hypothesized that the homeostasis of the Ins(1,4,5)P₃ level and the limited breakdown of PtdIns(4,5)P₂, only lasting approx. 6 h, is associated with the increase in 2,3-BPG acting *in vivo* as an inhibitor of inositol-polyphosphate 5-phosphatases. Vice versa, the observed delayed 2,3-BPG degradation in response to myo-inositol addition to starved cells may facilitate the rapid replenishment of the PtdIns(4,5)P₂ pool. A purified inositol-polyphosphate 5-phosphatase [22], highly expressed in the growth phase, with the characteristics of the recently cloned *Dd5P4* [9], was subjected to inhibition studies. It is a type II 5-phosphatase catalysing the dephosphorylation of soluble Ins(1,4,5)P₃ [less effective with Ins(1,3,4,5)P₄] and the membrane-bound PtdIns(4,5)P₂ [less effective with PtdIns(3,4,5)P₃]. The enzyme possess a *K_M* value of 40.2 ± 3.8 μM for Ins(1,4,5)P₃ and is inhibited by 2,3-BPG in a competitive manner [22]. After additional inhibition studies (Figure 8), the *K_i* was determined according to [46]. A *K_i* value of 40.6 ± 2.3 μM indicates that the substrate and the competitive inhibitor are bound with nearly equal affinities to this enzyme. The cellular concentrations of substrate and inhibitor as mentioned above suggest that this inhibition might be relevant *in vivo*. Further experiments are needed to confirm our hypothesis that 2,3-BPG variations influence cellular levels of signalling molecules by acting on the activity of 5-phosphatases. First of all, however, there are serious questions of how

these metabolic pathways are interconnected and what triggers the drastic 2,3-BPG increase as a consequence of the inositol deficiency.

Recently, a further unexpected link between glycolysis and *myo*-inositol metabolism was found in *S. cerevisiae* [47]. Mutants with a loss-of-function allele for triose phosphate isomerase exhibited inositol-auxotrophy and showed an 'inositolless death' phenotype. A proposed molecular explanation was presented; the mutants accumulate cellular dihydroxyacetone phosphate 30-fold over basal, which itself inhibits MIPS *in vitro*. Unfortunately, data for the cellular *myo*-inositol levels are lacking, and *myo*-inositol depletion was merely deduced from the observed inositol-auxotrophic phenotype. Nevertheless, considering these results and those presented here, there do exist unexplored interdependencies between glycolysis and *myo*-inositol metabolism. Further research on this topic may for instance explain why cellular alterations in inositol levels have been implicated in the aetiology of diabetic complications [48].

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