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**Disruption of inositol biosynthesis through targeted mutagenesis in *Dictyostelium discoideum* – Generation and characterisation of inositol-auxotrophic mutants**

Andreas Fischbach,<sup>1</sup> Stephan Adelt,<sup>1</sup> Alexander Müller and Günter Vogel<sup>2</sup>

Fachbereich C - Biochemie, Bergische Universität Wuppertal, Gausstrasse 20, D-42119 Wuppertal, Germany

<sup>1</sup> Both authors contributed equally to this publication.

<sup>2</sup> To whom correspondence should be addressed (phone +49-202-439-2791, fax +49-202-439-3399, e-mail [vogel@uni-wuppertal.de](mailto:vogel@uni-wuppertal.de)).

Short title: Inositol-auxotrophic mutants of *D. discoideum*

## SYNOPSIS

*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 D-*myo*-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 mold *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 media, the mutants lose their viability when deprived 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-bisphosphoglycerate, evidence could be found that the internal disturbances of inositol homeostasis triggers the accumulation. In a first attempt to characterise 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-bisphosphoglycerate is presented.

Key words: *Dictyostelium*, D-*myo*-Inositol 3-phosphate synthase, gene disruption, inositol polyphosphates, phosphoinositides, 2,3-bisphosphoglycerate

Abbreviations used: MIPS, D-*myo*-Inositol 3-phosphate synthase; FITC, fluorescein isothiocyanate; HPIC-IPAD, high-performance ion chromatography with integrated pulsed amperometric detection; HPIC-CD, high-performance ion chromatography with conductivity detection; MDD, metal dye detection.

## INTRODUCTION

Throughout the biological kingdom the cyclitol *myo*-inositol (Ins) and its metabolites are involved in diverse physiological processes such as growth regulation, membrane biogenesis and osmotolerance, apart from their significance in eucaryotic signal transduction. The biosynthesis of Ins follows an evolutionarily conserved pathway [1]. All Ins-producing organisms studied to date generate the cyclitol from glucose 6-phosphate (Glc6P) via an internal oxidoreduction and aldol cyclization reaction catalysed by D-*myo*-inositol 3-phosphate synthase (MIPS formerly known as L-*myo*-inositol 1-phosphate synthase; EC 5.5.1.4) in an NAD<sup>+</sup>-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 *S. cerevisiae* and has meanwhile been sequenced from evolutionarily diverse organisms. Deduced amino acid sequences reveal that eucaryotic MIPS are remarkably conserved throughout their length ( $\geq 45\%$  identity) and that the following stretches of amino acid residues are highly conserved: GWGGNNG, LWTANTERY, NGSPQNTFVPGL and SYNHLGNNDG. The GXGGXXG motif (Rossmann fold) is involved in NAD<sup>+</sup> 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 compounds, 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 mold prompted the NIH to select it as a model organism for functional analysis of sequenced genes ([http://www.nih.gov/science/models/d\\_discoideum/](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 eucaryotes, which are absent or less accessible in 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 involved [4, 5].

Ins is not an essential ingredient of well-defined culture media [6], so cells are able to synthesise the compound *de novo*. Evidence given below supports the involvement of MIPS.

Tritiated Ins transferred into the cytoplasm by electroporation is incorporated into phosphatidylinositol (PtdIns) within minutes [7]. Phosphorylation of PtdIns is accomplished by a family of five specific kinases (PIK 1-5), whose corresponding genes have been cloned [8]. Site-directed mutagenesis provokes defects in the organisation of the cytoskeleton and perturbations in differentiation. Certain double knockouts appear to 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 diacylglycerol and inositol 1,4,5-trisphosphate (Ins(1,4,5) $P_3$ ) 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 characterised [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 PtdIns(4,5) $P_2$ , PtdIns(3,4,5) $P_3$ , Ins(1,4,5) $P_3$  and Ins(1,3,4,5) $P_4$ . Further inositol phosphate metabolism seems to be very complex, with about 25 identified compounds in this organism and very little information about the enzymes involved [7]. Remarkable are the relatively large concentrations of Ins $P_6$  (about 0.3-0.6 mM) in the exponential growth phase while being cultured either on bacteria or in axenic media. Ins $P_6$  and the compounds containing energy-rich pyrophosphate groups, 6-PP-Ins $P_5$  (Ins $P_7$ ) and 5,6-bis-PP-Ins $P_4$  (Ins $P_8$ ), 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 fulfill 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 Ins $P_6$ ) in plant seeds. This should certainly be important for ancient organisms like amoebae, with a resistant stage in their life cycle.

A coordinated 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 Ins $P_6$ , Ins $P_7$  and Ins $P_8$ .

## EXPERIMENTAL

### Materials

All chemicals, buffers and media constituents used were reagent grade or better and were purchased by Sigma, Roth or Oxoid. Enzymes were obtained from Sigma, Roche or New England Bioscience. *myo*-[<sup>3</sup>H]Inositol, Hybond-N+ nylon membrane, Superdex 200 HR, Mono Q and Source 15Q anion exchange material were from Amersham Biosciences. 4-(2-Pyridylazo)resorcinol (PAR) was obtained from Fluka and YCl<sub>3</sub> 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. Ins(1,4,5)P<sub>3</sub> in cellular extracts was determined by the *D*-*myo*-Inositol 1,4,5-trisphosphate (IP<sub>3</sub>) [<sup>3</sup>H] Biotrak Assay System (# TRK1000, Amersham Biosciences) corresponding to the manufacturers recommendations. The reference substance Ins(1,4,5)P<sub>3</sub>, also used for the inhibition studies, was purchased as sodium salt from Sigma. All phosphoinositides were obtained from Echelon.

### Growth and development of *D. discoideum*

*D. discoideum* wild type cells (strain AX2, ATCC 24397) and the mutant strains described were grown axenically in synthetic FM medium [6] and semi-synthetic HL5 medium [11] under shaking (120 rpm, 22 °C) or submerge 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<sup>10</sup> cells/ml in 40 mM phosphate buffer, pH 6.8; 120 rpm, 22 °C) or in association with bacteria on SM agar plates [12]. Cells were harvested by centrifugation (500 g, 5 min, 4 °C). The cell density and the cell size of *D. discoideum* was determined with a Coulter<sup>®</sup> counter (model Z2). Chemotaxis was analysed by the agar-well assay [13]. Differentiation was initiated by plating washed exponentially growing cells on nonnutrient 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 *Eco*RI and *Hind*III and treated with T4-DNA polymerase to yield blunt ends. The fragment was inserted into a single *Asp*I 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 linearised with *Bam*HI, which increased homologous recombination efficiency.

### Antisense plasmid construction

A 1.5 kb *Bam*HI/*Sal*I fragment from FC-AA11 was inserted into pUC18 cleaved with *Bam*HI and *Sal*I. The 1.5 kb *Eco*RI/*Hind*III fragment from the resulting vector was cloned in antisense orientation in the *Eco*RI and *Hind*III 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<sub>2</sub>HPO<sub>4</sub>, 5 mM glucose). After centrifugation (500 g, 5 min, 4 °C) the cell pellet was resuspended in 800  $\mu$ l ice-cold electroporation buffer. 50  $\mu$ g DNA was added and the suspension was transferred in a 4 mm electroporation cuvette. Electroporation was performed with a single pulse (1.25 kV, 25  $\mu$ F, time constant: 0.5 ms) from the Gene Pulser (Bio-Rad).

Aliquots of the cell suspension (equal to  $1-5 \times 10^6$  cells) were added to 10 ml HL5 containing 500  $\mu$ M Ins and transferred to 80 mm Petri dishes. After 24 h, the medium was changed and the cells were kept in HL5 with 500  $\mu$ M Ins and 10  $\mu$ g/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 Ins were examined.

### Isolation of RNA and northern blot analysis

Total RNA is 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). 1.5  $\mu$ g RNA was size fractionated on a formaldehyde-containing agarose gel, transferred to a nylon membrane and probed with digoxigenin-UTP labelled RNA (DIG RNA Labelling Kit, Roche). Hybridised RNA species were detected with a DIG luminescent detection kit (Roche).

### Assay for D-myoinositol 3-phosphate synthase

20  $\mu$ l of the protein preparation was added to 80  $\mu$ l assay mixture (62.5 mM Mops, pH 8.0, 20 mM NH<sub>4</sub>Cl, 6.25 mM Glc6P, 1.25 mM NAD<sup>+</sup>) 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, 20 °C). Ins3P in the assay mixture was dephosphorylated by treatment with 5  $\mu$ l reaction buffer (50 mM Mops, pH 9.0, 80 mM MgCl<sub>2</sub>) and 4 U 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 deionised water and subjected to HPIC-IPAD.

### Cell viability

Cell viability could be determined by inoculating 4 ml HL5 medium supplemented with 500  $\mu$ M Ins in a tissue-culture flask (surface area: 25 cm<sup>2</sup>) with approximately 100 cells. Each viable cell led to a visible clone within 5-6 days. Other methods, 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 [16]. Pinocytosis was assayed as fluorescence internalised by *D. discoideum* cells incubated in phosphate buffer containing 2 mg/ml fluorescein-labelled dextran. Phagocytosis was measured as fluorescence incorporated by *D. discoideum* cells through uptake of FITC-labelled *E. coli* B/r.

### 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<sup>®</sup> MB-3, Merck) was determined by HPIC-IPAD (high-performance ion chromatography with integrated pulsed amperometric detection) 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). Excess glucose was eluted in a regeneration step with 1 M NaOH.

### Analysis of Ins3P, 2,3-BPG and glycolysis metabolites (HPIC-CD)

A perchloric acid extract of  $5 \times 10^8$  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 bisphosphates were eluted with 200 mM HCl. The resulting fraction was freeze-dried, diluted with H<sub>2</sub>O and subjected to HPIC-CD (high-performance ion chromatography with conductivity detection). The analysis was performed with a DX-500 ion chromatography system (DIONEX<sup>®</sup>) consisting of a gradient pump (GP50), a chromatography oven (LC30) with an internal valve bearing a 100  $\mu$ 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 x 50 mm, DIONEX<sup>®</sup>) precolumn and an IonPac AS11-HC (4 x 250 mm, DIONEX<sup>®</sup>) analytical column



were used. To separate the inositol monophosphates the column was eluted isocratically with 10.5 mM KOH (water/MeOH 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-bisphosphoglycerate was purified by HPLC (Mono Q eluted with HCl-gradient: see inositol phosphate analytics) from a perchloric acid extract of  $10^9$  *Ddino1Δ1* cells preincubated 24 h on a medium without inositol. After freeze-drying this method yielded 50-75 nmol 2,3-BPG. The homogeneity of the compound was confirmed as described below. 30 nmol 2,3-BPG was subjected to a 4 h enzymatic degradation with 250 mU phosphoglycerate mutase (EC 5.4.2.1, Sigma). The reaction was carried out at room temperature in 1.5 ml 75 mM Bistris, 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; fructose 1,6-bisphosphate, 31.7 min; 2,3-bisphosphoglycerate, 37 min).

### **Analysis of phosphoinositides (HPIC-CD)**

Phosphoinositides were extracted with 9.5 ml  $\text{CHCl}_3/\text{MeOH}/12 \text{ M HCl}/\text{H}_2\text{O}$  (3:4:0.5:1 by vol.) from  $2 \times 10^9$  cells. The mixture was incubated at room temperature (30 min, ultrasonic bath) and 3.75 ml  $\text{CHCl}_3$  and 3.75 ml  $\text{H}_2\text{O}$  were subsequently added. After vortexing and centrifugation (5000 g, 10 min, 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  $\text{CHCl}_3$ . 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 a RP/H Maxi-Clean Cartridge (Alltech). Aliquots of  $2 \times 10^7$  cells were used for analysis. To elute glycerophosphoinositol and the glycerophosphoinositol phosphates we used a water/MeOH 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 coeluted 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 to Ins.

Freeze-dried samples derived from  $5 \times 10^7$  cells were dissolved in 400  $\mu$ l H<sub>2</sub>O, and 50  $\mu$ l 90 mM NaIO<sub>4</sub> was added to oxidise the glycerol backbone (20 min, rt, darkness). Excess NaIO<sub>4</sub> was destroyed by addition of 20  $\mu$ l 700 mM Na<sub>2</sub>SO<sub>3</sub> (20 min, rt, darkness). 40  $\mu$ l dimethylhydrazine (1 % (w/v) adjusted to pH 4.5 with formic acid) was added, and the reaction mixture was incubated for 4 h to convert the resulting glycolaldehyde to Ins1P. Ins1P was dephosphorylated by treatment with 1 ml 50 mM Mops, pH 9.0 and 10 U alkaline phosphatase for 4 h at 37 °C. The reaction was stopped by heating at 95 °C for 2 min. The samples were deionised on a mixed bed ion exchanger (Amberlite<sup>®</sup> MB-3, Merck), and inositol was quantified by HPIC-IPAD.

#### **Analysis of inositol phosphates (HPLC-MDD) and ATP**

Perchloric acid 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). InsP<sub>6</sub>-InsP<sub>8</sub> 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 postcolumn derivatisation with a metal-dye reagent (2 M Tris/HCl, pH 9.1, 200  $\mu$ M 4-(2-pyridylazo)resorcinol (PAR), 30  $\mu$ M YCl<sub>3</sub>, 10 % (v/v) MeOH; 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<sup>®</sup> 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, 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  $\approx$  50 mU/mg) and utilised 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  $\mu$ l of buffer (160 mM Bistris, pH 7.0, 800 mM sucrose, 1 mM EDTA, 20 mM MgCl<sub>2</sub>), 10  $\mu$ l of water or a solution of the inhibitor 2,3-BPG (0.03-2 mM), 10  $\mu$ l of the corresponding protein fraction, and 10  $\mu$ l of 0.64 mM Ins(1,4,5)P<sub>3</sub> 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://dictyadb.biol.tsukuba.ac.jp/>) and the *D. discoideum* genomic database (<http://dictybase.org/>) were screened for putative MIPS homologues containing the conserved motifs (see Introduction). A cDNA (FC-AA11) of about 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.

A stringent requirement for generating vital inositol-auxotrophic mutants is the uptake of external Ins that will bypass the deficiency in inositol biosynthesis. Our experiments and previous studies [24] monitoring the incorporation of *myo*-[<sup>3</sup>H]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 exist. Southern blot analysis of genomic DNA digested with restriction enzymes not present in the cDNA sequence of FC-AA11 detected only one hybridising fragment with a <sup>32</sup>P-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 (*Ddino1* $\Delta$ 1 - *Ddino1* $\Delta$ 3) and 18 independent transformants by antisense mutagenesis (*Ddino1as*1 - *Ddino1as*18). Two strains (*Ddino1* $\Delta$ 1 and *Ddino1as*1) showing the most pronounced effects on MIPS expression were

selected for further studies. Their molecular defects were confirmed by northern blots and enzyme activity assays (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 characterisation of MIPS from the parent strain AX2 (for details on HPIC-IPAD and the enzyme characteristics see Supplementary data).

## Phenotypic characterisation 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 Ins for optimal growth, indicating that endogenous biosynthesis is adequate. In contrast the mutants *Ddino1Δ1* and *Ddino1as1* are indeed inositol-auxotrophic and grow only after Ins supplementation. At concentrations < 100 μM Ins no growth of *Ddino1Δ1* is detectable (Figure 2). In the presence of 500 μM Ins the growth rates of the mutants are similar to those of AX2 cells. Where not otherwise noted, the phenotypic and metabolic changes are demonstrated exemplarily 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 Ins free medium, *Ddino1Δ1* cells completely stop growing, become smaller and lose their typical amoeboid form within 24 hours (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 internalised 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 growth after Ins 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 Ins. Shatkin und 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 bacteria 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 procaryotes contain no Ins. It seems puzzling that, in contrast to the growth defect observed on axenic media, 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 Ins supply may cause a disturbed inositol phosphate/phosphoinositide turnover with potential impacts on vital processes, as utilisation 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 Ins was examined. Independently of the substrate chosen, gene inactivation does not affect chemotaxis (data not shown). In comparison to the parent strain, plated on nonnutrient 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 needs 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 PI3-kinases (DdPIK1 and DdPIK2) closely related to mammalian p110 PI3-kinase [8]. Another report describes the identification of a developmentally regulated PI4-kinase that is highly active in post-aggregate stages [31]. Altogether, these data 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 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 Ins, spores are also capable of germinating, but the resulting cells die within the next few hours. In the presence of Ins, 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 phagocytosis**

Pinocytosis is the exclusive route for entry of nutrients into cells cultured on liquid media [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 internalisation 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 Ins, phagocytosis of *Ddino1Δ1* cells is comparable to that of the parent strain. No significant difference was observed for the internalisation 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 Ins starvation the uptake rate of bacteria and the phagocytosis capacity of the cells are reduced. After 24 h Ins starvation the cells completely lose their ability to phagocytose (Figure 5B).

In contrast to 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 phago-lysosomes. It is well known that phosphoinositides are required for the internalisation process in a number of cell types [32, 33] and that they play a role in phagosomal maturation in *D. discoideum* [30]. This aspect will be taken up again considering the results of the metabolite analytics.

### **Alterations in inositol-containing metabolites**

#### **Water-soluble compounds**

Soluble inositol metabolites of *Ddino1Δ1* were analysed after 24 hours of inositol starvation. At the beginning of the experiment cellular levels of the common precursor inositol are usually high, varying between 350 and 600  $\mu\text{M}$ , 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 Ins to the fluid media, lies in the range 40-60  $\mu\text{M}$ . We assume that this steady-state level is optimal for vital functions in an exponentially growing culture. In the mutant only 3-4  $\mu\text{M}$  are preserved after 24 h incubation on inositol-free media (Table 1), confirming that MIPS indeed catalyses the committed and rate-limiting step in the *de novo* synthesis of Ins.

The level of  $\text{Ins}3P$ , the actual product of the conversion of  $\text{Glc}6P$  by MIPS, was determined by HPIC-CD. It is maintained between 0.6-1.0  $\mu\text{M}$ . It should be noted that an achiral method was used, and the enantiomer  $\text{Ins}1P$  could be present as well. Furthermore other metabolic pathways might lead to the formation of  $\text{Ins}1P$  and  $\text{Ins}3P$ .

An isotope dilution assay was used to quantify  $\text{Ins}(1,4,5)P_3$ . No significant alteration of the  $\text{Ins}(1,4,5)P_3$  concentration (0.6-0.8  $\mu\text{M}$ ) occurs after inositol starvation.

As found for plant seeds, some highly phosphorylated inositol phosphates, particularly inositol hexakisphosphate, may fulfill storage functions and serve as a unimolecular source of Ins, phosphate and also metal ions on account of their complexation properties [34]. Typical intracellular concentrations of  $\text{Ins}P_6$  (up to 600  $\mu\text{M}$ ),  $\text{Ins}P_7$  (up to 100  $\mu\text{M}$ ) and  $\text{Ins}P_8$  (up to 300  $\mu\text{M}$ ) in *D. discoideum* [10] are high enough, that their complete dephosphorylation could compensate 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  $\text{Ins}P_6$  concentration tends to decrease, whereas  $\text{Ins}P_7$  and  $\text{Ins}P_8$  concentrations increases. Taking into account that the mean cellular volume of the mutants is smaller, especially after Ins 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  $\text{Ins}P_6$  pool is actually decomposed, but the phenotypic changes and the inositol analytics indicate that this is not enough to compensate the inositol demand. Various explanations are conceivable for why such great concentrations of the highly phosphorylated inositol phosphates are conserved. A simple explanation may be that their metabolism is too lethargic to meet the needs for inositol in vegetative growth. From metabolic labelling studies of amoebae with [ $^3\text{H}$ ]Ins it is known that the marker accumulates relatively slowly in the compounds  $\text{Ins}P_6$ ,  $\text{Ins}P_7$  and  $\text{Ins}P_8$  [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 *Ddino1A1* cells are inositol-starved for 24 h (Figure 3). In contrast the cells die afterward, 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 a 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  $\text{Ins}P_6$ -kinase abolished the synthesis of  $\text{Ins}P_7$  and  $\text{Ins}P_8$  and resulted in a strain with accelerated aggregation but unimpaired differentiation [37], so it is unlikely that the pyrophosphorylated compounds are significantly involved.  $\text{Ins}P_6$  or the pentakisphosphates

remain to be considered. We are currently studying an inositol pentakisphosphate 3/5-kinase [38] that participates in  $\text{InsP}_6$ -homeostasis, and hope to obtain further hints 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 coelution 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-bisphosphoglycerate (2,3-BPG). Both *Ddino1Δ1* cells and those of the antisense mutant *Ddino1as1*, which were generated by a completely different method that targets *ino1* expression, accumulate 2,3-bisphosphoglycerate under inositol starvation (the effect on cellular 2,3-BPG for *Ddino1as1* is about  $\frac{1}{3}$  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, Ins supplementation to the media enhanced the steady-state PtdIns level significantly (*Ddino1Δ1* and AX2 (500  $\mu\text{M}$  Ins)  $\approx$  100-125  $\mu\text{M}$ , AX2 (without supplementation)  $\approx$  60-70  $\mu\text{M}$ ), whereas the steady-state levels of all phosphatidylinositol phosphates investigated were unchangeable by addition of Ins. After 24h starvation the PtdIns level of *Ddino1Δ1* decreased clearly below the basal level to approximately 5  $\mu\text{M}$  (Table 1). Phosphoinositide biosynthesis starts with the formation of PtdIns from Ins and CDP-DAG catalysed by PtdIns synthase (EC 2.7.8.11), so it seems consistent that cellular inositol and phosphatidylinositol concentrations are both affected. A comparable influence of inositol starvation on phosphatidylinositol was reported for inositol-auxotrophic cells of *N. crassa* [26], *S. cerevisiae* [39] and *CHO* 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 phosphatidylinositol was compensated by phosphatidylglycerol [27], which would help to retain the cell dimensions. Despite additional two-dimensional thin layer chromatography we were unable to detect such a shift in phospholipid composition (data not shown). Accordingly, inositol-starved *Ddino1Δ1* cells become smaller, and it can be hypothesised that the disturbance in membrane biogenesis is associated with the early cessation of cytokinesis.

PtdIns is the exclusive precursor for all phosphatidylinositol phosphates 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 Ins(1,4,5) $P_3$  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 phosphatidylinositol phosphates, especially of PtdIns(3,4,5) $P_3$ . It can be concluded that their concentrations are clearly below 0.2  $\mu$ M. In comparison to PtdIns the intracellular levels of the phosphatidylinositol phosphates investigated are not as strongly affected. It seems that a mechanism exists that stabilises 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 only last temporarily. Therefore after 24 hours re-synthesis of phosphatidylinositol phosphates 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 a 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-bisphosphoglycerate - 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 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 fructose 1,6-bisphosphate and ATP concentrations, to exclude a metabolic dysfunction that results secondarily in a 2,3-BPG increase. Neither the phosphofruktokinase product nor ATP underlies significant fluctuations in the first 24 h (*Ddino1Δ1* (+) or *Ddino1Δ1* (-): Fru(1,6) $P_2$  = 254 ± 22  $\mu$ M, ATP = 935 ± 96  $\mu$ M comparable to data presented in [42]).

There is at least one ancient function of 2,3-BPG in glycolysis that makes it indispensable for all eucaryotic 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 a physiological relevance of this inhibition was postulated

[43]. There are hints of a reciprocal action: that inositol phosphates, especially  $\text{Ins}(1,4,5)\text{P}_3$ , interact with aldolase [44], and that the  $\text{Ins}(1,4,5)\text{P}_3$  receptor associates with glyceraldehyde 3-phosphate dehydrogenase [45]. These literature data encouraged us to analyse the time course of the changes in cellular inositol,  $\text{Ins}(1,4,5)\text{P}_3$  and phosphoinositide concentrations in relation to the 2,3-BPG concentration, as provoked by Ins starvation in the first 24 h. This might be an indicator of a causal relationship. *Ddino1Δ1* cells cultured on synthetic media supplemented with 500  $\mu\text{M}$  Ins show artificially high cellular Ins 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 Ins levels were still slightly higher than the basal level of the parent strain (40–60  $\mu\text{M}$ ). The intracellular Ins concentration of the mutant falls very far below this value in the first two hours (Figure 7A). Afterwards it decreases only slightly, reaching a plateau of about 3–4  $\mu\text{M}$  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 2–3  $\mu\text{M}$ . Coinciding with a deficit in cellular Ins, after 2 h the level rises fourfold and then continuously to a maximum at 14 h, when it remains at a level up to fifty times that of basal. In contrast, the cellular  $\text{Ins}(1,4,5)\text{P}_3$  concentration fluctuates only within the error margins over the whole 22 hours (0.6–0.8  $\mu\text{M}$  nearly equal to  $[\text{Ins}(1,4,5)\text{P}_3]_i$  in AX2 see Table 1). On starvation the PtdIns level decreased below the basal value in the first 2 hours, and the decline comes to rest after about 14 hours at a low level of approximately 6  $\mu\text{M}$ . Thus the time dependencies of the Ins and the PtdIns concentrations show striking similarities in the progression and the minimal value finally reached, which is in both cases about 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 (data not shown). The level of the  $\text{Ins}(1,4,5)\text{P}_3$  precursor PtdIns(4,5) $\text{P}_2$  decreases for 6 hours to approximately 1/3 of the initial value with a slight tendency to recover afterwards. Of the metabolites that vary, the PtdIns(4,5) $\text{P}_2$  level normalises fastest after a renewed Ins supplementation. It needs only 4 h, followed by PtdIns and 2,3-BPG (about 8 h, data not shown).

We hypothesised that the homeostasis of the  $\text{Ins}(1,4,5)\text{P}_3$  level and the limited breakdown of PtdIns(4,5) $\text{P}_2$ , only lasting about 6 hours, 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 Ins addition to starved cells may facilitate the fast

replenishment of the PtdIns(4,5) $P_2$  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) $P_3$  (less effective with Ins(1,3,4,5) $P_4$ ) and the membrane-bound PtdIns(4,5) $P_2$  (less effective with PtdIns(3,4,5) $P_3$ ). The enzyme possess a  $K_M$ -value of  $40.2 \pm 3.8 \mu\text{M}$  for Ins(1,4,5) $P_3$  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  $40.6 \pm 2.3 \mu\text{M}$  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 Ins 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 Ins levels are lacking, and Ins 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 Ins metabolism. Further research on this topic may for instance explain why cellular alterations in inositol levels have been implicated in the etiology of diabetic complications [48].

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## REFERENCES

- 1 Majumder, A. L., Chatterjee, A., Dastidar, K. G. and Majee, M. (2003) Diversification and evolution of L-*myo*-inositol 1-phosphate synthase. *FEBS Lett.* **553**, 3-10
- 2 Majumder, A. L., Johnson, M. D. and Henry, S. A. (1997) 1L-*myo*-Inositol-1-phosphate synthase. *Biochim. Biophys. Acta* **1348**, 245-256
- 3 Kuspa, A., Sucgang, R. and Shaulsky, G. (2001) The promise of a protiste: the *dictyostelium* genome project. *Funct. Integr. Genomics* **1**, 279-293
- 4 Irvine, R. F. and Schell, M. J. (2001) Back in the water: the return of the inositol phosphates. *Nat. Rev. Mol. Cell. Biol.* **2**, 327-338
- 5 Cullen, P. J., Cozier, G. E., Banting, G. and Mellor, H. (2001) Modular phosphoinositide-binding domains – their role in signalling and membrane trafficking. *Curr. Biol.* **11**, R882-R893
- 6 Franke, J. and Kessin, R. (1977) A defined minimal medium for axenic strains of *Dictyostelium discoideum*. *Proc. Natl. Acad. Sci. U.S.A.* **74**, 2157-2161
- 7 Van Haastert, P. J. M. and Van Dijken, P. (1997) Biochemistry and genetics of inositol metabolism in *Dictyostelium*. *FEBS Lett.* **410**, 39-43
- 8 Zhou, K., Takegawa, K., Emr, S. D. and Firtel, R. A. (1995) A phosphatidylinositol (PI) kinase gene family in *Dictyostelium discoideum*: biological roles of putative mammalian p110 and yeast VPs34p PI 3-kinase homologs during growth and development. *Mol. Cell. Biol.* **15**, 5645-5656
- 9 Loovers, H. M., Veenstra, K., Snippe, H., Pesesse, X., Erneux, Ch. And Van Haastert, P. J. M. (2003) A diverse family of inositol 5-phosphatases playing a role in growth and development in *Dictyostelium discoideum*. *J. Biol. Chem.* **278**, 5652-5658
- 10 Laussmann, T., Pikzack, C., Thiel, U., Mayr, G. W. and Vogel, G. (2000) Diphospho-*myo*-inositol phosphates during the life cycle of *dictyostelium* and *polysphondylium*. *Eur. J. Biochem.* **267**, 2447-2451
- 11 Watts, D. J. and Ashworth, J. M. (1970) Growth of myxameobae of the cellular slime mould *Dictyostelium discoideum* in axenic culture. *Biochem. J.* **119**, 171-174
- 12 Sussman, M. (1987) Cultivation and synchronous morphogenesis of *dictyostelium* under controlled experimental conditions. In *Methods in Cell Biology* **28** (Spudich, J. A., ed.), pp. 9-29, Academic Press, NY
- 13 Wallace, L. J. and Frazier, W. A. (1979) Photoaffinity labeling of cyclic-AMP- and AMP-binding proteins in differentiating *Dictyostelium discoideum* cells. *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4250-4254

- 14 Adachi, H., Hasebe, T., Yoshinaga, K., Ohta, T. and Sutoh, K. (1994) Isolation of *Dictyostelium discoideum* cytokinesis mutants by restriction enzyme-mediated integration of the blasticidin S resistance marker. *Biochem. Biophys. Res. Commun.* **205**, 1808–1814
- 15 Faix, J., Gerisch, G. and Noegel, A. A. (1992) Overexpression of the csA cell adhesion molecule under its own cAMP-regulated promoter impairs morphogenesis in *dictyostelium*. *J. Cell Sci.* **102**, 203–214
- 16 Vogel, G., Thilo, L., Schwarz, H. and Steinhart, R. (1980) Mechanism of phagocytosis in *Dictyostelium discoideum*: phagocytosis is mediated by different recognition sites as disclosed by mutants with altered phagocytotic properties. *J. Cell Biol.* **86**, 456-465
- 17 Podeschwa, M., Plettenburg, O., vom Brocke, J., Block, O., Adelt, S. and Altenbach, H. J. (2003) Stereoselective synthesis of *myo*-, *neo*-, *L-chiro*-, *D-chiro*-, *allo*-, *scyllo*-, and *epi*-inositol systems via conduritols prepared from *p*-benzoquinone. *Eur. J. Org. Chem.* **10**, 1958-1972
- 18 Clarke, N. G. and Dawson, R. M. (1981) Alkaline O → N-transacylation. A new method for the quantitative deacylation of phospholipids. *Biochem. J.* **195**, 301-306
- 19 Nasuhoglu, C., Feng, S., Mao, J., Yamamoto, M., Yin, H. L., Earnest, S., Barylko, B., Albanesi, J. P. and Hilgemann, D. W. (2002) Nonradioactive analysis of phosphatidylinositides and other anionic phospholipids by anion-exchange high-performance liquid chromatography with suppressed conductivity detection. *Anal. Biochem.* **301**, 243-254
- 20 Mayr, G. W. (1988) A novel metal-dye-detection system permits picomolar-range h.p.l.c. analysis of inositol polyphosphates from non-radioactively labelled cell or tissue specimens. *Biochem. J.* **254**, 585-591
- 21 Adelt, S., Plettenburg, O., Stricker, R., Reiser, G., Altenbach, H. J. and Vogel, G. (1999) Enzyme-assisted total synthesis of the optical antipodes *D-myo*-inositol 3,4,5-trisphosphate and *D-myo*-inositol 1,5,6-trisphosphate: aspects of their structure-activity relationship to biologically active inositol phosphates. *J. Med. Chem.* **42**, 1262-1273
- 22 Adelt, S. (1999) Untersuchungen zum Stoffwechsel von *myo*-Inositolphosphaten in *Dictyostelium discoideum* – Anreicherung und Charakterisierung von Phosphohydrolasen. In *Berichte aus der Biologie* (ISBN 3-8265-6760-9), Shaker Verlag, Aachen
- 23 Van Lookeren Campagne, M. M., Erneux, C., Van Eijk, R. and Van Haastert, P. J. M. (1988) Two dephosphorylation pathways of inositol 1,4,5-trisphosphate in homogenates of the cellular slime mould *Dictyostelium discoideum*. *Biochem. J.* **254**, 343-350

- 24 Europe-Finner, G. N., Gammon, B. and Newell, P. C. (1991) Accumulation of [<sup>3</sup>H]-inositol into inositol polyphosphates during development of *dictyostelium*. *Biochem. Biophys. Res. Commun.* **181**, 191-196
- 25 Henry, S. A., Atkinson, K. D., Kolat, A. I. and Culbertson, M. R. (1977) Growth and metabolism of inositol-starved *Saccharomyces cerevisiae*. *J. Bacteriol.* **130**, 472-484
- 26 Hanson, B. and Brody, S. (1979) Lipid and cell wall changes in an inositol-requiring mutant of *Neurospora crassa*. *J. Bacteriol.* **138**, 461-466
- 27 Jackowski, S., Voelker, D. R. and Rock, C. O. (1988) Inositol metabolism and cell growth in a chinese hamster ovary cell *myo*-inositol auxotroph. *J. Biol. Chem.* **263**, 16830-16836
- 28 Shatkin, A. J. and Tatum, E. L. (1961) The relationship of m-inositol to morphology in *Neurospora crassa*. *Am. J. Bot.* **48**, 760-771
- 29 Clarke, M. and Kayman, S. C. (1987) The axenic mutations and endocytosis in *Dictyostelium*. In *Methods in Cell Biology* **28** (Spudich, J. A., ed.), pp. 157-176, Academic Press, NY
- 30 Rupper, A. C., Rodriguez-Paris, J. M., Grove, B. D. and Cardelli, J. A. (2001) p110-related PI 3-kinases regulate phagosome-phagosome fusion and phagosomal pH through a PKB/Akt dependent pathway in *Dictyostelium*. *J. Cell Sci.* **114**, 1283-1295
- 31 Varela, I., Van Lookeren Campagne, M. M., Alvarez, J. F. and Mato, J. M. (1987) The developmental regulation of phosphatidylinositol kinase in *Dictyostelium discoideum*. *FEBS Lett.* **211**, 64-68
- 32 Botelho, R. J., Teruel, M., Dierckman, R., Anderson, R., Wells, A., York, J. D., Meyer, T. and Grinstein, S. (2000) Localized biphasic changes in phosphatidylinositol-4,5-bisphosphate at sites of phagocytosis. *J. Cell Biol.* **151**, 1353-1367
- 33 Cox, D., Tseng, C. C., Bjekic, G. and Greenberg, S. (1999) A requirement for phosphatidylinositol 3-kinase in pseudopod extension. *J. Biol. Chem.* **274**, 1240-1247
- 34 Stevenson-Paulik, J., Bastidas, R. J., Chiou, S.-T., Frye, R.A. and York, J. D. (2005) Generation of phytate-free seeds in *Arabidopsis* through disruption of inositol polyphosphate kinases. *Proc. Natl. Acad. Sci. U.S.A.* **102**, 12612-12617
- 35 Frederick, J. P., Mattiske, D., Wofford, J. A., Megosh, L. C., Drake, L. Y., Chiou, S.-T., Hogan, B. L. M. and York, J. D. (2005) An essential role for an inositol polyphosphate multikinase, *Ipk2*, in mouse embryogenesis and second messenger production. *Proc. Natl. Acad. Sci. U.S.A.* **102**, 8454-8459

- 36 Verbsky, J., Lavine, K. and Majerus, Ph. W. (2005) Disruption of the mouse inositol 1,3,4,5,6-pentakisphosphate 2-kinase gene, associated lethality, and tissue distribution of 2-kinase expression. *Proc. Natl. Acad. Sci. U.S.A.* **102**, 8448-8453
- 37 Luo, H. R., Huang, Y. E., Chen, J. C., Saiardi, A., Iijima, M., Ye, K., Huang, Y., Nagata, E., Devreotes, P. and Snyder, S. H. (2003) Inositol polyphosphates mediate chemotaxis in *Dictyostelium* via pleckstrin homology domain-PtdIns(3,4,5) $P_3$  interactions. *Cell* **114**, 559-572
- 38 Stephens, L. R., Hawkins, Ph. T., Stanley, A. F., Moore, T., Poyner, D. R., Morris, P. J., Hanley, M. R., Kay, R. R. and Irvine, R. F. (1991) *myo*-Inositol pentakisphosphates – structure, biological occurrence and phosphorylation to *myo*-inositol hexakisphosphate. *Biochem. J.* **275**, 485-499
- 39 Becker, G. W. and Lester, R. L. (1977) Changes in phospholipids of *Saccharomyces cerevisiae* associated with inositol-less death. *J. Biol. Chem.* **252**, 8684-8691
- 40 Weeks, G., and Herring, F. G. (1980) The lipid composition and membrane fluidity of *Dictyostelium discoideum* plasma membranes at various stages during differentiation. *J. Lipid Res.* **21**, 681-686
- 41 Siddhanta, A., Radulescu, A., Stankewich, M. C., Morrow, J. S. and Shields, D. (2003) Fragmentation of the golgi apparatus – a role for  $\beta$ III spectrin and synthesis of phosphatidylinositol 4,5-bisphosphate. *J. Biol. Chem.* **278**, 1957-1965
- 42 Martinez-Costa, O. H., Estevez, A. M., Sanchez, V. and Aragon, J. J. (1994) Purification and properties of phosphofructokinase from *Dictyostelium discoideum*. *Eur. J. Biochem.* **226**, 1007-1017
- 43 Rana, R. S., Chandra Sekar, M., Hokin, L. E. and MacDonald, M. J. (1986) A possible role for glucose metabolites in the regulation of inositol-1,4,5-trisphosphate 5-phosphomonoesterase activity in pancreatic islets. *J. Biol. Chem.* **261**, 5237-5240
- 44 Thieleczek, R., Mayr, G. W. and Brandt, N. R. (1989) Inositol polyphosphate-mediated repartitioning of aldolase in skeletal muscle triads and myofibrils. *J. Biol. Chem.* **264**, 7349-7356
- 45 Patterson, R. L., Van Rossum, D. B., Kaplin, A. I., Barrow, R. K. and Snyder, S. H. (2005) Inositol 1,4,5-trisphosphate receptor/GAPDH complex augments  $Ca^{2+}$  release via locally derived NADH. *Proc. Natl. Acad. Sci. U.S.A.* **102**, 1357-1359
- 46 Kulhavy, D., Cegan, A., Komers, K. and Mindl, J. (2002) Inhibition of enzymatic reactions. A rapid method to determine the index  $pI_{50}$ . *Z. Naturforsch.* **57c**, 496-499

- 47 Shi, Y., Vaden, D. L., Ju, S., Ding, D., Geiger, J. H. and Greenberg, M. L. (2005) Genetic perturbation of glycolysis results in inhibition of *de novo* inositol biosynthesis. *J. Biol. Chem.* **280**, 41805-41810
- 48 Fisher, S. K., Novak, J. E. and Agranoff, B. W. (2002) Inositol and higher inositol phosphates in neural tissues: homeostasis, metabolism and functional significance. *J. Neurochem.* **82**, 736-754



## TABLES AND FIGURES

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

To calculate the concentrations following mean cell volumes were used: 751 ± 20 fl (AX2), 606 ± 19 fl (*Ddino1Δ1* (+)) and 513 ± 16 fl (*Ddino1Δ1* (-)). Presented data are the mean ± S.D. of five independent experiments.

| <b>C<sub>i</sub> [μM]</b>    | <b>AX2 (-)</b> | <b><i>Ddino1Δ1</i> (+)</b> | <b><i>Ddino1Δ1</i> (-)</b> |
|------------------------------|----------------|----------------------------|----------------------------|
| <i>myo</i> -Ins              | 45 ± 5         | 483 ± 175                  | 3.8 ± 1.4                  |
| Ins3P                        | 0.6 ± 0.2      | 1 ± 0.3                    | 0.8 ± 0.2                  |
| 2,3-bisphosphoglycerate      | 6.1 ± 0.9      | 3 ± 0.7                    | 135 ± 18                   |
| Ins(1,4,5)P <sub>3</sub>     | 0.58 ± 0.11    | 0.80 ± 0.14                | 0.73 ± 0.14                |
| Ins(1,2,3,4,6)P <sub>5</sub> | 15.2 ± 3.2     | 12.1 ± 3                   | 22 ± 3                     |
| Ins(1,2,4,5,6)P <sub>5</sub> | 22.1 ± 3       | 18 ± 2                     | 28.3 ± 4                   |
| InsP <sub>6</sub>            | 660 ± 35       | 513 ± 48                   | 454 ± 45                   |
| InsP <sub>7</sub>            | 88 ± 17        | 76 ± 16                    | 96 ± 21                    |
| InsP <sub>8</sub>            | 264 ± 29       | 375 ± 39                   | 441 ± 39                   |
| PtdIns                       | 64 ± 7         | 125 ± 15                   | 5.4 ± 1.2                  |
| PtdIns4P                     | 5.3 ± 0.4      | 4.1 ± 0.5                  | 4.7 ± 0.3                  |
| PtdIns3P                     | 3.5 ± 0.4      | 5 ± 0.9                    | 3.8 ± 0.4                  |
| PtdIns(4,5)P <sub>2</sub>    | 7.5 ± 0.9      | 6 ± 0.5                    | 2.3 ± 0.3                  |

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

(A) A blot containing 1.5  $\mu\text{g}$  RNA in each lane isolated from vegetative cells cultured on FM medium in the presence of 500  $\mu\text{M}$  Ins (+) or 24 h without Ins (-) was probed with digoxigenin-UTP labelled complementary RNA. (B) MIPS activity comparison between AX2 and the mutants. The data are presented as the mean  $\pm$  S.D. of three independent experiments.

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

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

**Figure 3 Photographs of vegetative cells and two differentiation stages of *Ddino1Δ1* and for comparison those of the parent strain AX2**

**Figure 4 Survival of *Ddino1Δ1* after sustained inositol deficiency**

The data are presented as the mean  $\pm$  S.D. of three independent experiments.

**Figure 5 Fluid-phase uptake and phagocytosis of bacteria**

(A) Comparison of fluid-phase uptake of AX2 ( $\blacktriangle$ ) with *Ddino1Δ1* in the presence of 500  $\mu\text{M}$  Ins ( $\blacksquare$ ) and after 24 h Ins starvation ( $\square$ ). Data are referred to surface area. *Ddino1Δ1* (-) cells needed a lag-phase of about 30 min to recover from the washing procedure. (B) Uptake of FITC-labelled *E. coli* B/r by AX2 ( $\blacktriangle$ ) and *Ddino1Δ1* at different times of Ins starvation (0 h,  $\blacksquare$ ; 8 h,  $\circ$ ; 16 h,  $\Delta$ ; 24 h,  $\square$ ).

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

(A) Separation of metabolites with up to five phosphate groups extracted from  $2 \times 10^8$  *Ddino1Δ1* cells cultured on FM medium in the presence of 500  $\mu\text{M}$  Ins (+) or from an equal amount of cells incubated 24 h without Ins (-) and (B) An analogues experiment with the extract from  $1 \times 10^7$  cells and a gradient separating  $\text{InsP}_6$ ,  $\text{InsP}_7$  and  $\text{InsP}_8$ .

**Figure 7 Kinetics of the concentrations of selected inositol metabolites and 2,3-BPG in the strain *Ddino1A1* subjected to inositol starvation**

(A) Water-soluble inositol-containing compounds: Ins(1,4,5) $P_3$  ( $\square$ ), 2,3-BPG ( $\bullet$ ) and *myo*-Ins ( $\blacktriangle$ ). (B) Phosphatidylinositol and phosphatidylinositol phosphates: PtdIns ( $\blacksquare$ ), PtdIns4P ( $\circ$ ) and PtdIns(4,5) $P_2$  ( $\Delta$ ). Data are from one experiment, which is representative of at least three experiments.

**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) $P_3$  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 vs. concentration of the inhibitor at constant initial substrate and enzyme concentration results in a linear curve. The  $K_i$ -value for 2,3-BPG was calculated from the slope, as described in [46].

Figure 1

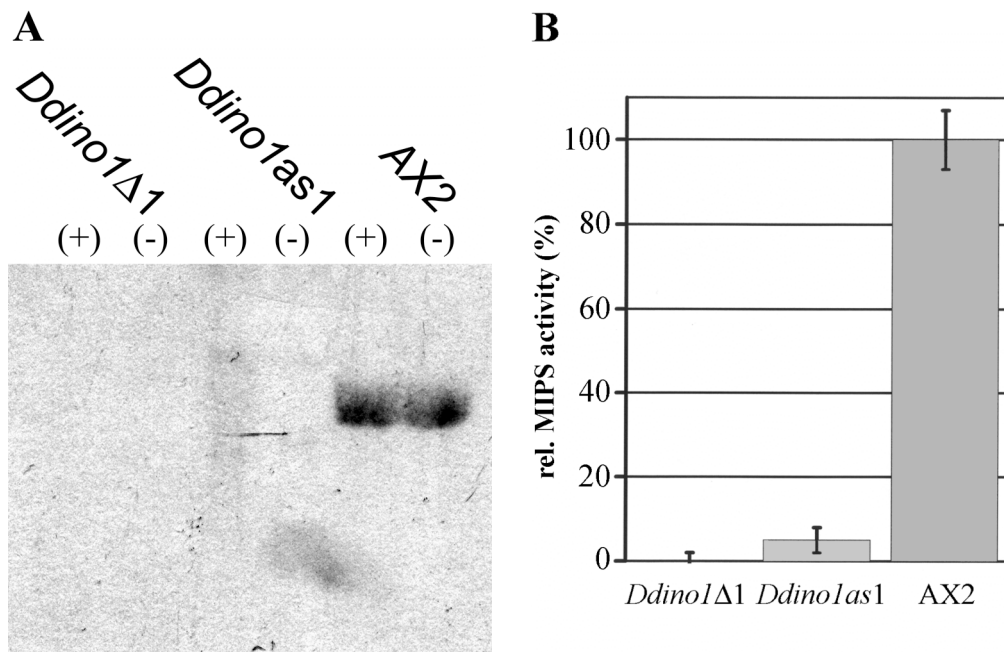


Figure 2

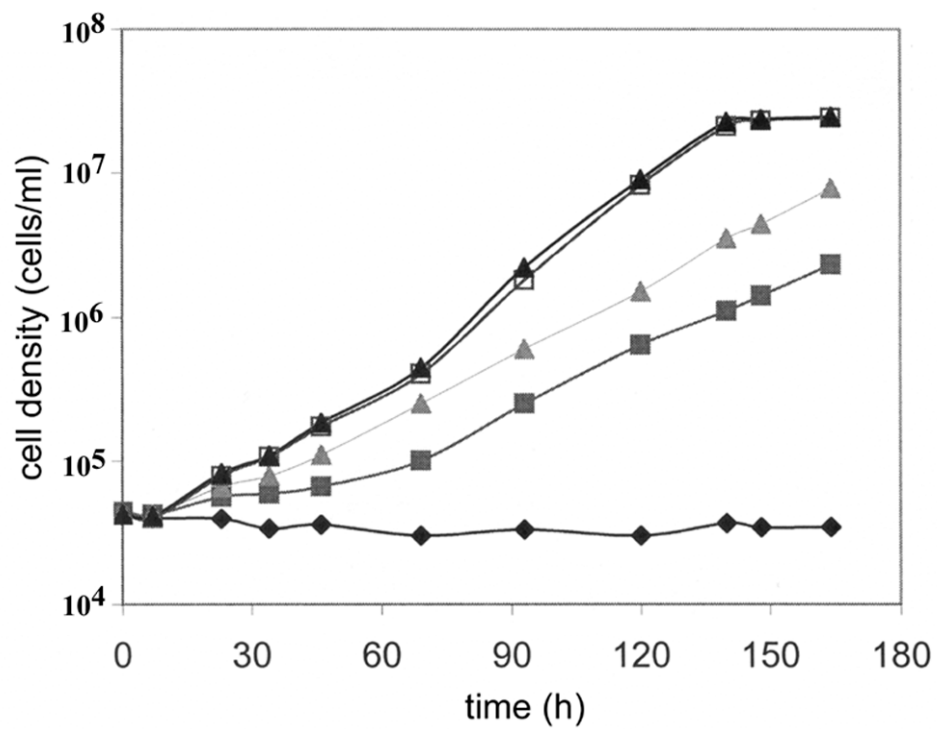


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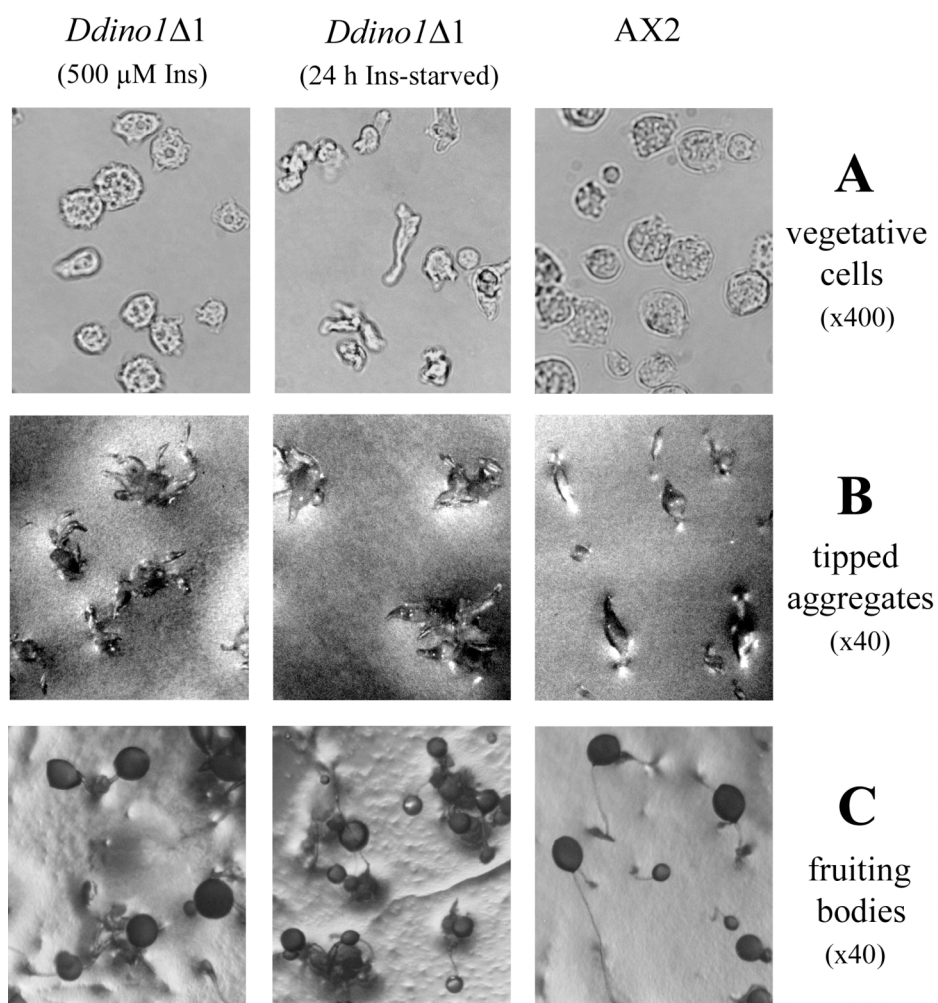


Figure 4

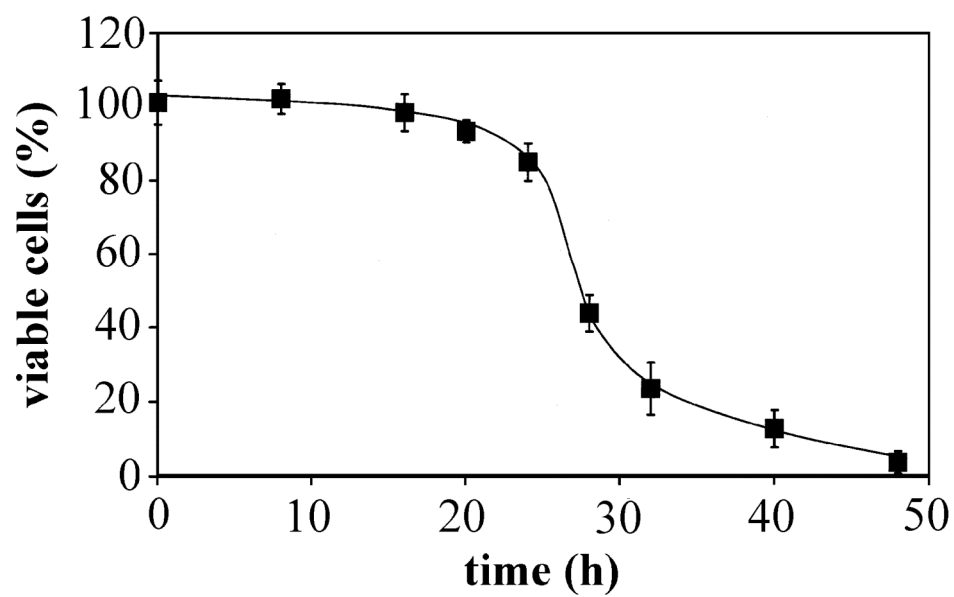


Figure 5

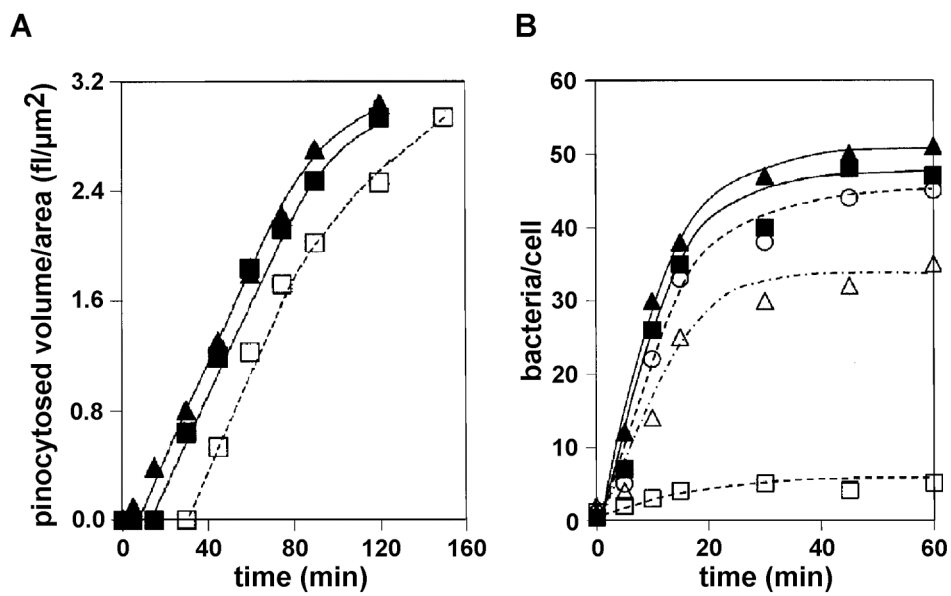




Figure 6

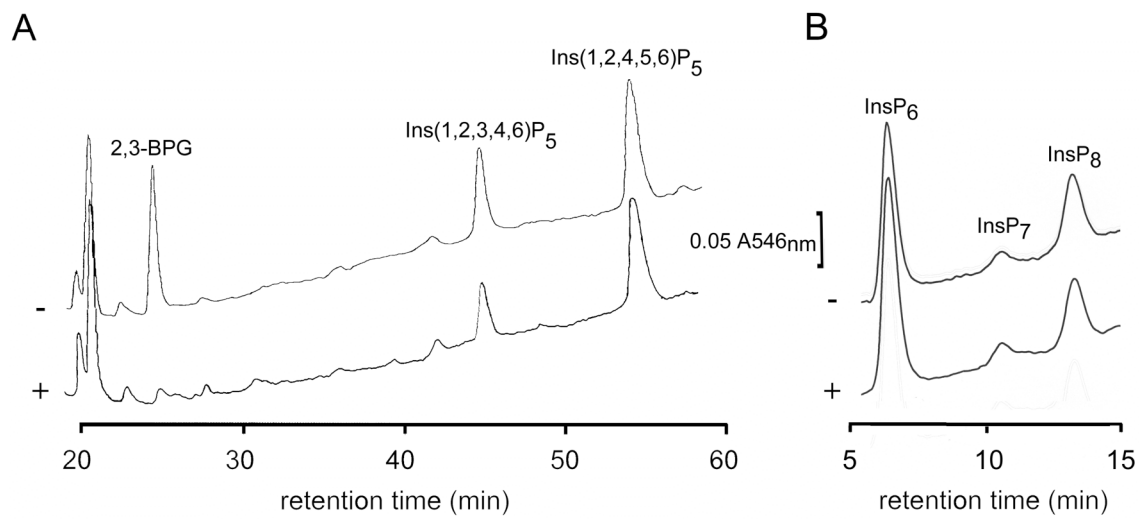


Figure 7

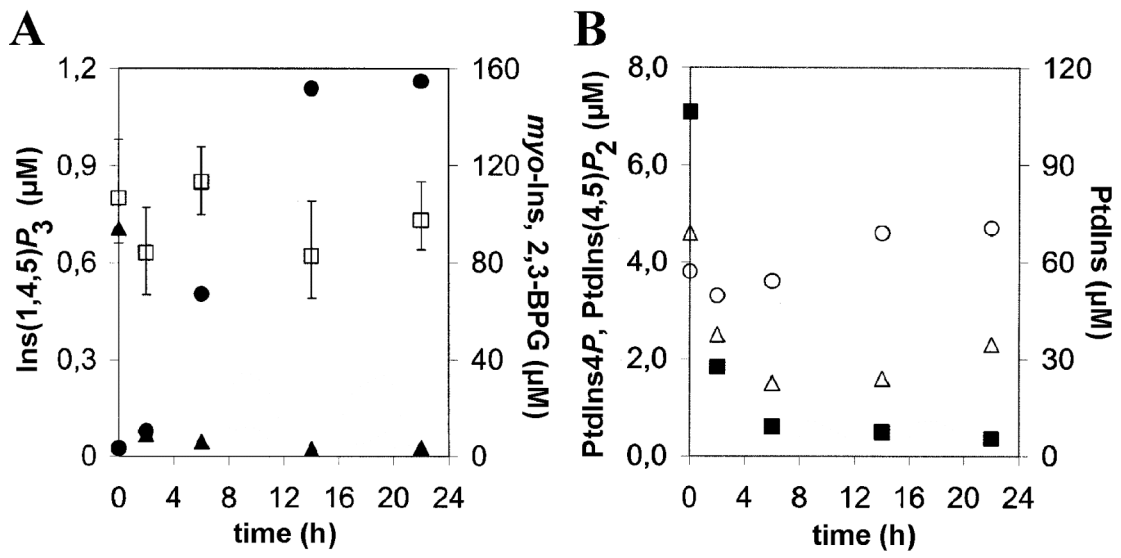


Figure 8

