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# Use of the giant multinucleate plasmodium of *Physarum polycephalum* to study RNA interference in the myxomycete

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**Abstract**

The plasmodium of *Physarum polycephalum* harbors billions of synchronized nuclei in a single cell of complex structure. Due to its synchrony and extreme size, it is used as a model to study events on a single cell level, such as cell cycle and differentiation. We show here for the first time that this model, despite its enormous size and structural complexity, is accessible to RNA interference by simple injection of dsRNA or siRNA. The targeted gene is that of polymalatase, an intracellular adapter of poly(β-L-malate) involved in the maintenance of the synchrony and functioning as an extracellular hydrolase of this polymer. Real-time reverse transcriptase polymerase chain reaction analysis revealed that the specific mRNA was knocked down to about 10% of the original level. The suppression of a single injection lasted for approximately 14 cell cycles (144 h) and could be prolonged for any time by repeated dsRNA injections. Western blots indicated that the knockdown of RNA was paralleled by a strong reduction in polymalatase synthesis. However, a change in the phenotype of the plasmodium could not be clearly observed. In principle, the plasmodium offers an easy system for studying gene knockdown by RNA interference.


*Keywords:* RNA interference; Polymalatase; Polynucleate cells; Plasmodium; Microinjection

*Physarum polycephalum* is one of the plasmodium-forming slime molds [1] of the class Myxomyceteae, a member of the monophylum Mycetozoa, and is more closely related to animals and fungi than to plants [2]. The plasmodium is a unique organism, representing a giant vegetative single cell in the life cycle of this slime mold. Its nuclei divide every 7–8 h in high synchrony, without dissolution of their nuclear envelope and in the absence of cytokinesis, thus giving rise to a giant multinucleate cell. The macroplasmodium (routinely in sizes of 100 $cm^2$ or larger) grows easily in the laboratory as a flat, disk-like cell on the surface of a solid support. Because of its large size, the plasmodium has evolved devices to allow the intracellular equilibration of cellular consitituents: (1) a complex macroscopic network of veins consisting of a rhythmically moving endoplasm surrounded by a resting ectoplasm, which displays a complicated structure of fibrils and invaginations of the plasma membrane [3], and (2) a (putative) molecular transporter consisting of polymalate and an adapter [4 and references therein]. By adopting a structural similarity to the backbone of nucleic acids, polymalate binds nuclear proteins, and the adapter guides the polymalate–cargo complex to the various nuclei. In the extracellular matrix, secreted adapter functions as hydrolase (polymalatase) of extracellular polymalate.

The plasmodium has been found suitable for studying various cellular and organismic aspects such as cell cycle,

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motility, and differentiation. Timely questions are the molecular basis of the synchrony [5] or the complex intracellular network recruiting commitment and development of sporulation [6,7]. The plasmodium is superior to any other known model organism by virtue of its enormous cellular size suitable for injections of materials such as nucleic acids or other materials [8], the high synchrony of cellular events [9], the fusibility of individual plasmodia allowing the transfer of molecular information [6,10], and the availability of both haploid and diploid species [10] to study gene dominance. However, attempts to knockout or overexpress genes by conventional techniques were mostly unsuccessful, rendering *P. polycephalum* an unsuccessful model system.

To overcome this problem, the goals of our investigation were (1) to demonstrate that, in contrast to its large size and complex structure, the plasmodium was accessible to RNA interference by simple injection, (2) to assess whether repeated injections give rise to enhanced suppression effects, (3) to measure the duration of the achieved RNAi effect, (4) to test whether the effect continued after replating of plasmodia, (5) to examine whether the effect was reversible, and (6) to examine whether, in the present case of the knockdown of polymalatase, a change in phenotype was detectable.

## Materials and methods

### *dsRNA and siRNA*

The DNA template to be transcribed into dsRNA was synthesized by PCR using the cDNA derived from *P. polycephalum* M3CVII plasmodia. Desalted primers specific for the polymalatase–cDNA (Accession No. AJ543320) with the sequences 5′-GTG TAA TAC GAC TCA CTA TAG GGA AAA GGA GGT TCT GAT CCT AGT-3′ (forward primer) and 5′-CAC TAA TAC GAC TCA CTA TAG GGA TCA CGA TGT CAT CAG CAA AAC-3′ (reverse primer), both containing the T7-polymerase promoter at their 5′ termini, were custom-made by MWG-Biotech, Germany. The resulting 589-bp DNA spanned the nucleotides 1083–1671 of the gene downstream of the origin of transcription and was used as template for in vitro dsRNA synthesis as described by Donze and Picard [11]. The 50-μl reaction mixture was incubated for 15 h and then treated for 30 min with RNAse-free DNase (Qiagen, Germany) at 37 °C. The mixture was finally heated to 95 °C for 5 min and cooled to room temperature over a period of 7 h. Double-stranded RNA was precipitated with 2.5 volumes of ethanol/0.2 M sodium acetate, pH 4.9, pelleted at 20,000*g* for 20 min at 4 °C, washed with 70% ethanol, air-dried, and resuspended in 60 μl of diethylpyrocarbonate-treated RNase-free water. The integrity of the dsRNA was validated by agarose gel electrophoresis in Tris–acetate–EDTA-buffer, pH 8.0, before aliquots of 10 μl were stored at −80 °C. For control injections, nonspecific dsRNA was generated by the same method using a PCR-derived fragment with 592-bp (nucleotides 142–734) from the vector pGEM(R)-5zf(+) (Technical Services, Promega, USA).

To synthesize siRNA, a motif based on the polymalatase gene (Accession No. AJ543320) was designed according to the outlines proposed by Elbashir et al. [12] employing the advanced RNAi software (oligoengine internet homepage). The templates 5′-TCG AGT AAG TAC TAG AGC TCC TAT AGT GAG TCG TAT TAG T-3′ (containing the sense segment) and 5′-AAG GAG CTC TAG TAC TTA CTC TAT AGT GAG TCG TAT TAG T-3′ (containing the antisense segment) were annealed with the T7-promotor segment 5′-ACT AAT ACG ACT CAC TAT AG-3′ for RNA synthesis in the presence of T7-RNA polymerase according to the method of Donze and Picard [11]. The resulting 19-bp RNA duplex contained 2 nt overhangs essential for the function of siRNA containing UU in the sense strand [13]. The template for transcription of nonspecific control siRNA had the sequences 5′-AAG GCG GTA ATA CGG TTA TCC-3′ as sense and 5′-GTG GAT AAC CGT ATT ACC GCC-3′ as antisense strands. All DNA synthetic work was carried out by MWG, Germany.

### *Macroplasmodia and injection of dsRNA*

Microplasmodia of *P. polycephalum* strain M3CVII ATCC 204388 (American Type Culture Collection) were cultured by the method of Daniel and Baldwin [14] for 48 h at 24 °C. To start a macroplasmodium, 100 mg of wet microplasmodia were allowed to fuse on 2% agar (13.5 cm petri dish) containing the growth medium and cultured 22 h at 24 °C in the dark. The macroplasmodium (5 ± 0.5 cm in diameter) was injected into a prominent vein with 10 μl of a solution [8] containing 1 μg of dsRNA, siRNA, or control RNAs and allowed to grow for 24 h before the plasmodium was scraped from the agar surface into liquid nitrogen and stored at −80 °C for analysis.

In long-term experiments, macroplasmodia were grown and injected the same way, but 24 h after injection a 4-$cm^2$ section of the macroplasmodium together with the agar was cut out and placed as an inoculum upside down on a fresh agar plate (first generation). The remainder of the plasmodium was scraped from the agar plate and analyzed. The inoculum was allowed to grow for 60 h, receiving fresh injections of dsRNA after 24 and 48 h. Then a section was transferred for replating, the remainder of the plasmodium was prepared for analysis (after 60 h from replating) as above, and so on until three generations had been injected and prepared for analysis. In a second series, the plasmodium was injected only in the parental plasmodium, and the following generations