# An Immunological Approach to Enrich a Mitotic Stimulator and to Reveal $G_2$-Phase-specific Proteins in *Physarum polycephalum*

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ABSTRACT Purified antibodies from an antiserum against S-phase proteins of the myxomycete *Physarum polycephalum* were attached to protein-A-Sepharose CL-4B. A late $G_2$-phase extract that contained a mitosis-stimulating protein was applied to this immunoadsorbent, and the mitosis-stimulating protein was enriched by a factor of ten. This protein, which is present in the cell in low amounts, is synthesized in late $G_2$ phase and obviously degraded in a later stage of the cycle. Immunoadsorption of a $G_2$-phase extract with anti-S-antibodies decreased the 700 main proteins to 20 as demonstrated by two-dimensional gel electrophoresis. No difference in protein pattern could be observed on two-dimensional gels between S-phase and $G_2$-phase extracts before and after immunoadsorption with anti-S-antibodies. This indicates that there are no $G_2$-phase-specific proteins among the 700 most abundant proteins of *Physarum polycephalum*.

There are several reports in the literature that deal with the periodical synthesis of proteins during the cell cycle. These investigations were performed in various organisms, e.g., in *E. coli* (12). No protein synthesized at different rates during parts of the cell cycle could be identified. In several eucaryotic organisms, like *Saccharomyces cerevisiae* (6), *Physarum polycephalum* (4, 9, 21) or HeLa cells (3, 14), certain proteins are synthesized at different rates during the cell cycle. Among the few variable proteins, tubulins have been identified in HeLa cells (3) as well as in *Physarum polycephalum* (4, 9).

Apart from periodic synthesis through the cell cycle, the question remains whether there are specific proteins that are only present at a distinct phase of the cell cycle. There are conflicting results with respect to this question. In *E. coli* (12) and in *Saccharomyces cerevisiae* (6), no such proteins could be detected. In HeLa cells, Al-Bader et al. (1) found phase-specific proteins. However, Bravo and Celis (3) disproved these latter findings, since they could not detect any phase-specific proteins in HeLa cells. In *Physarum polycephalum*, only one yet unidentified cycle-dependent protein present during mitosis was found (18). We could not find significant differences in protein pattern on two-dimensional gels when we compared extracts from the S and $G_2$ periods (Gröbner, P., and P. Loidl, unpublished results). To facilitate the evaluation of two-dimensional gels, we tried to reduce the number of spots by immunoadsorption of the most abundant proteins with antibodies.

The detection of cell cycle-specific proteins was of special interest for us, since we have obtained previous evidence for the existence of a mitotic stimulator (10). This factor was only present at a distinct time in the $G_2$ period of the cell cycle of *Physarum polycephalum*. It is likely that this stimulator is a protein (10). We tried to enrich this mitotic protein by immunoadsorption of the main cellular proteins on anti-S-antibodies attached to protein-A-Sepharose CL-4B. We used anti-S-antibodies because this immunoadsorption should only retain those $G_2$-phase proteins that are not phase specific and therefore present during the entire interphase.

We have chosen *Physarum polycephalum* as a model system for this investigation because *Physarum* offers the unique advantage of a naturally synchronous system in which mitosis occurs ~ every 10 h. Furthermore, we have previously introduced a bioassay to test for mitotic stimulators by direct measurement of mitotic acceleration (10). Other investigators, who claim to have found mitotic factors (19, 20, 23), tested the induction of mitosis-specific events (e.g., nuclear membrane breakdown or chromatin condensation in amphibian oocytes) but could not quantitate the mitosis-inducing effect.

## MATERIALS AND METHODS

*Culture Strains and Preparation of Plasmodial Extracts:* We used the strain $M_3b$, a Wis 1 derivative. Macroplasmodia were cultivated in petri dishes on filter paper supported by glass beads on a sterile semi-defined nutrient medium (5) supplemented with 0.013% hemoglobin instead of he-



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matin. Mitosis was determined in ethanol-fixed smears under a phase-contrast microscope (7). Entire plasmodia were harvested at selected stages of the nuclear division cycle and stored at −30°C. Frozen samples were thawed, suspended in 1 ml (for immunization) or 2 ml of 0.02 M Tris-HCl buffer (pH 7.2 at 25°C), homogenized by sonication (MSE Ltd., Crawley, Sussex, UK; low power, amplitude 1; 3 times, each for 5 s at 0°C), and centrifuged for 30 min at 30,000 *g*. Supernatants were used for immunization, immunoadsorption, two-dimensional gel electrophoresis, determination of accelerating capacity on mitosis as described earlier (10), and protein analysis (11). For two-dimensional gel electrophoresis and determination of accelerating effect, plasmodial extracts were diluted 1:2 with 0.02 M Tris-HCl buffer.

*Antibody Preparation and Purification of Physarum Extracts by Immunoadsorption:* Antiserum that contained antibodies against *Physarum* proteins from early S-phase plasmodia (0.5 h after mitosis 3) was produced in four rabbits by five multiple intradermal injections, 1 wk apart, each 8-mg extract protein in 1 ml together with 1 ml of Freund's complete adjuvant. 1 wk after the last immunization, the blood was obtained by heart puncture and allowed to clot at 37°C for 1 h. After inactivation of complement at 56°C for 30 min, the pooled sera were stored at −30°C. Antibodies were isolated from aliquots of thawed antiserum by salt precipitation (three times with ammonium sulfate at 35% saturation), and then fractionation on DE-52-diethylaminoethyl-cellulose (Whatman Biochemicals Ltd., Springfield Mill, Kent, UK) was repeated twice, as described (8). The isolated antibodies were dialyzed against 0.1 M phosphate buffer (pH 7.0 at 25°C) for 4 h and stored in aliquots at −30°C.

1 ml protein-A-Sepharose CL-4B (Pharmacia, Inc., Uppsala, Sweden) was suspended in 0.1 M phosphate buffer, pH 7.0, and thawed antibodies (30 mg/ml gel) were added to a final volume of 10 ml. The suspension was shaken gently for 1 h at room temperature and then washed twice with 0.1 M phosphate buffer, pH 7.0, and several times with 0.02 M Tris-HCl buffer, pH 7.2, on a glass filter until all unbound protein was eluted. 1 ml gel (binding capacity was 25 mg IgG/ml gel) with the bound IgG-type antibodies was transferred to 1 ml *Physarum* extract (1.25 mg *Physarum* protein/ml extract). The mixture was shaken for 1 h at room temperature and then filtered through a glass filter; the gel was washed twice with 0.5 ml 0.02 M Tris-HCl buffer, pH 7.2. The combined effluents (2 ml) were analyzed for protein content, accelerating capacity on mitosis, and by two-dimensional gel electrophoresis in parallel with the 1:2 diluted untreated *Physarum* extract. The protein-A-Sepharose CL-4B was regenerated by washing the gel five times with 0.58% (vol/vol) glacial acetic acid in 0.15 M NaCl and five times with 0.1 M phosphate buffer, pH 7.0.

*Two-Dimensional Gel Electrophoresis:* Two-dimensional gel electrophoresis was performed as described by O'Farrell (15) with some modifications. Plasmodial extracts or immunoadsorption eluates were made 5% (vol/vol) 2-mercaptoethanol, 9 M urea, and 3% (wt/vol) Ampholine, pH 3.5–10 (LKB Produkter AB, Bromma, Sweden). Cylindrical gels (0.3 × 13.5 cm) were made of 5% (wt/vol) acrylamide, 0.15% (wt/vol) *N, N'*-methylenebisacrylamide, 2% (vol/vol) Nonidet P-40, 0.02% (wt/vol) ammoniumperoxodisulfate, 0.07% (wt/vol) *N, N, N', N'*-tetramethylethylenediamine, 1.5% (wt/vol) Ampholine (pH 3.5–10), and 1.5% (wt/vol) Ampholine (pH 5–8). 100-μl samples (not more than 50 μg protein/gel) were applied to the anode and overlaid with 25 μl of a 6 M urea and 3% (wt/vol) Ampholine (pH 3.5–10) mixture. The gels were run at 10°C with constant voltage at 400 V for 1 h, then 1 h at 600 V, and finally 6 h at 1,000 V (7,000 V·h). The gels were frozen in dry ice, and the pH gradient was determined by slicing a gel into 4-mm sections, incubating them with 1 ml of distilled water for 2 h, and measuring the pH at 10°C with a glass electrode. The first dimension gels were equilibrated for 1 h in 10 ml equilibration buffer (15). The second dimension sodium dodecyl sulfate slab gel (0.075 × 14 × 14 cm) consisted of a 12% polyacrylamide separating gel overlaid by a 5% polyacrylamide stacking gel. After electrophoresis, gels were stained with silver stain (13).

## RESULTS

Antibodies against *Physarum* proteins from early S phase (mitosis + 0.5 h) were raised in rabbits as described in Materials and Methods. 1 wk after the last immunization, antibody levels rose to a titer of 1:64 as estimated by double immunodiffusion (17). To characterize the antiserum, the purified antibodies were attached to protein-A-Sepharose CL-4B, and an S-phase extract was applied to this immunoadsorbent with an antibody/extract protein ratio of 20:1. After immunoadsorption, the eluant was compared with an untreated S-phase extract by two-dimensional gel electrophoresis (Fig. 1, *a* and *b*). The number of spots was decreased dramatically by immunoadsorption from ~700 to 20, which indicated the presence of antibodies against almost all S-phase proteins.

We have previously shown that a mitosis-stimulating protein is only present in late $G_2$ phase (10). With the antibodies against S-phase proteins, we tried to enrich this protein by immunoadsorption of $G_2$-phase proteins. We expected that the only proteins that should remain are those either present only in $G_2$ phase or against which no antibodies have been produced. Fig. 1 *c* shows a two-dimensional gel of a $G_2$-phase extract treated with anti-S-antibodies. There are no differences in the protein pattern when compared with an antibody-treated S-phase extract. This experiment shows that there are no late $G_2$-phase–specific proteins among the 700 main cellular proteins.

However, the antibody-treated late $G_2$-phase extract completely retained its mitosis-stimulating activity, although it contained <10% of the original protein content (Table I). As a control, we treated the late $G_2$-phase extract with antibodies from the serum of the rabbits before the first immunization. Table I shows that the extract after this type of immunoadsorption also retained the mitosis-stimulating activity, but the protein content of the extract was only slightly decreased. As an additional control, an S-phase extract was also tested before and after immunoadsorption. No pronounced accelerating effect on mitosis could be observed. Furthermore, we tested a premitotic extract before and after immunoadsorption. The slight effect on mitosis was not changed by immunoadsorption as compared with the untreated extract.

## DISCUSSION

The results reported in this paper are in agreement with our earlier observation (10) that a mitosis-stimulating protein is only present at a distinct point in late $G_2$ period. We were able to enrich this protein by a factor of ten (in terms of specific activity) by immunoadsorption of the majority of proteins. Oppenheim and Katzir (16) also found a mitosis-stimulating activity in late $G_2$-phase homogenates of *Physarum*. In contrast, Blessing and Lempp (2) found a slight stimulating activity on mitosis (maximum 6%) only in extracts of premitotic plasmodia (0.75 h before mitosis) after treatment with anti-S-antibodies. Untreated extracts could not accelerate mitosis. We also find a slight stimulating activity in premitotic extracts, but in contrast to Blessing and Lempp (2), immunoadsorption of such an extract with anti-S-antibodies did not increase the stimulating effect on mitosis (Table I). Blessing and Lempp (2) concluded that anti-S-antibodies remove mitosis-inhibiting factors. They suggested that the mitosis-stimulating protein is in some way repressed after its maximum point of activity. According to their repression theory, an earlier time point in $G_2$ phase should exist in which the accelerating effect is equal before and after treatment with anti-S-antibodies. Unfortunately, their report (2) does not contain information about such an experiment. We cannot approve a repression theory, since we could not increase the mitogenic effect of premitotic extracts by immunoadsorption.

We suggest that the mitosis-stimulating protein is synthesized during a distinct period of the cell cycle and is degraded rapidly thereafter. Other investigators (19, 20, 23), who measured cell cycle–specific cytoplasmic factors capable of inducing mitosis-specific events (e.g., nuclear membrane breakdown, chromatin condensation), came to similar conclusions. Our assumption of a cyclic production of a mitosis-inducing

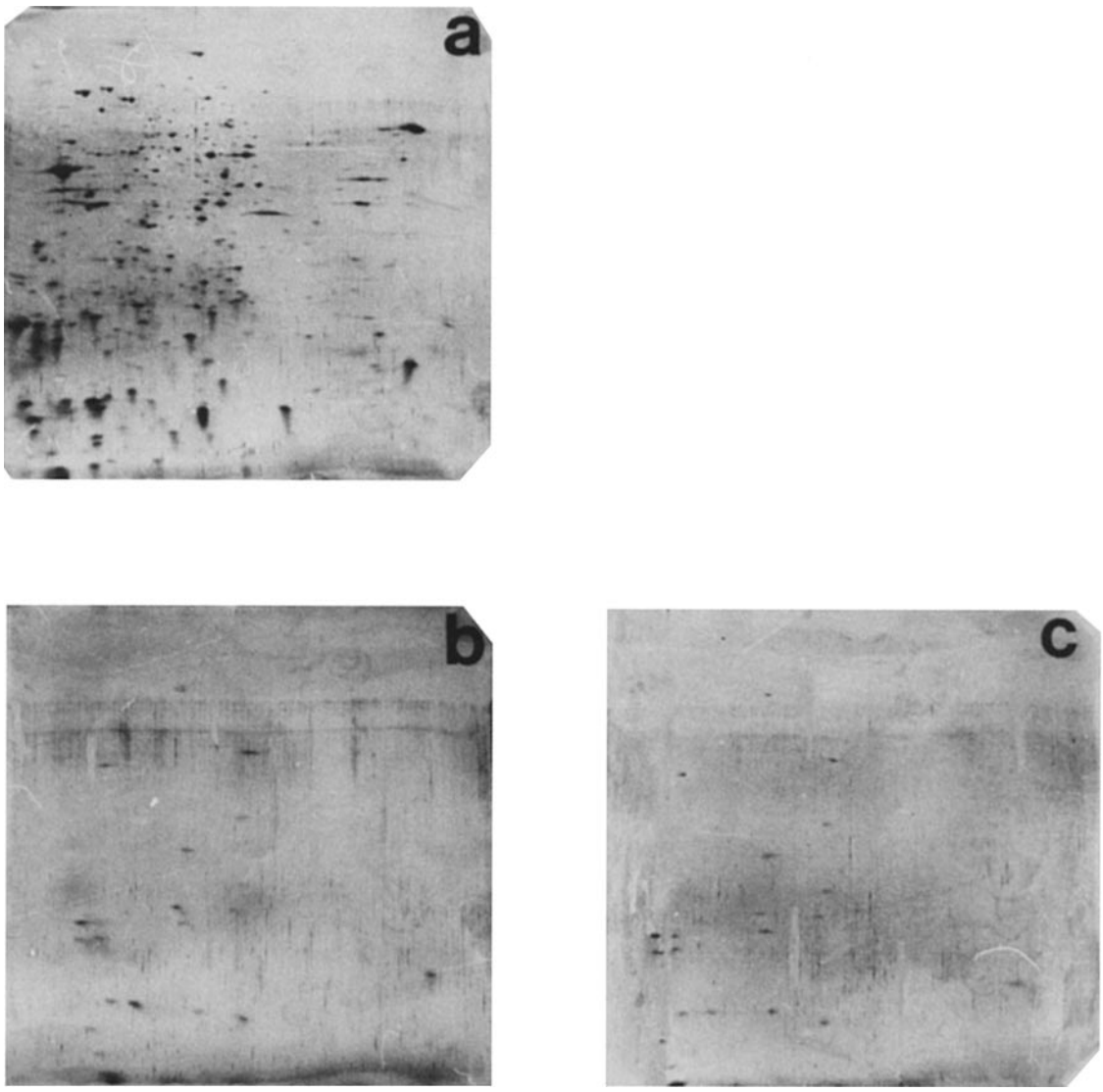


FIGURE 1 Silver-stained two-dimensional gels of extracts from macroplasmodia of *Physarum polycephalum*. (a) Plasmodial extract from S phase (mitosis 2 + 0.5 h; 41.3 μg protein); (*b*) the same extract after immunoadsorption (3.8 μg protein); (c) plasmodial extract from late $G_2$ phase (mitosis 2 + 7.5 h) after immunoadsorption (4.1 μg protein). The biological properties of these preparations are described in Table I. The pH gradient in the first dimension (isoelectric focusing) was from 4.4 at anode (left side in the figure) to pH 8.0 at the cathode end (right side); the second dimension (sodium dodecyl sulfate gel electrophoresis) separated proteins with molecular weights from ~10,000 to 150,000 and was performed as described in Materials and Methods.

TABLE I

*Immunoadsorption and Accelerating Effect on Mitosis of Plasmodial Extracts from Selected Stages of the Cell Cycle*

| | Before immunoadsorption | | After immunoadsorption | | | |
|---|---|---|---|---|---|---|
| Stage of the cell cycle‡ | Protein | Acceleration of mitosis 1‖ | Type of immunoadsorption§ | Protein | Residual protein | Acceleration of mitosis 1‖ |
| | μg/ml | % | | μg/ml | % | % |
| S phase (mitosis 2 + 0.5 h) | 625 | 5.6 ± 2.9* | A | 55 ± 12.1 | 8.8 ± 2.0 | 6.4 ± 3.6* |
| late $G_2$ phase (mitosis 2 + 7.5 h) | 625 | 20.3 ± 2.8 | A | 60 ± 13.9 | 9.6 ± 2.2 | 18.1 ± 3.8 |
| | | | B | 403 ± 46.8 | 64.5 ± 7.5 | 19.7 ± 3.2 |
| premitotic time (mitosis 2 + 9.0 h) | 625 | 5.9 ± 2.4* | A | 65 ± 16.9 | 10.4 ± 2.7 | 6.7 ± 2.8* |

Plasmodial extracts from selected stages of the cell cycle were subjected to immunoadsorption and analyzed before and after immunoadsorption for protein content and accelerating effect on mitosis 1 in recipient test plasmodia, as described in Materials and Methods. Each value represents the mean with a standard deviation for seven independent experiments, except *B* (three experiments).

* Significantly different ($P < 0.001$) versus late $G_2$ phase in Student's *t* tests for paired as well as grouped data.

‡ Cell cycle time (mitosis 2 − mitosis 3) was 10.1 h.

§ *A*, immunoadsorption on anti-S-antibodies. *B*, control, immunoadsorption on unspecific antibodies obtained before first immunization.

‖ Control cell cycle time of recipient test plasmodia (time from addition of culture medium until mitosis 1) was 7.6 ± 0.29 h.

protein is supported by recent data of Wagenaar (22), who investigated this question by a different approach. With the protein synthesis inhibitor emetine, he could show that the synthesis of mitotic proteins is restricted to a distinct period of the cell cycle in sea urchin embryos. His observations also indicate that mitotic proteins lose their activity at the end of mitosis, and new synthesis of these proteins is required for every cell cycle.

The question remains why we could not detect the mitosis-inducing protein at the time of its highest activity in late $G_2$ phase on our two-dimensional gels. This could be due to the very low amount of this protein below our detection level, since we can only visualize the 700 main proteins of a cell. It suggests that such a protein, which is synthesized and degraded periodically in a short part of the cell cycle, is only present in low amounts. Another explanation could be that the protein is either very basic or very acidic and therefore not within the pI range in the isoelectric focusing step (4.4–8.0).

Other groups, who investigated cell cycle–dependent proteins to look for mitotic factors in various cell systems, also failed to detect cell cycle phase-specific proteins (6, 12, 14). In contrast, Al-Bader et al. (1) reported that HeLa cells in S phase differ from $G_2$-phase cells by the absence of at least 25 proteins among a total of 150 spots as visualized by Coomassie Blue staining. Bravo and Celis (3) disproved these data. They could not detect any cell cycle phase-specific proteins in HeLa cells among 700 proteins as revealed by fluorography.

However, the main problem of such investigations on phase-specific proteins is the distinct detection of proteins on two-dimensional gels which contain up to 1,000 spots. The usual way for the evaluation of such two-dimensional gels is to compare the complex protein patterns from different cell cycle phases. Even if this is performed by the aid of a computer, it is a rather hopeless test. We tried a new alternative approach by first reducing the great number of spots by immunoadsorption of the main cellular proteins. The few remaining spots on such a two-dimensional gel can be more easily evaluated. With this method we could show that there are no differences between S-phase and $G_2$-phase protein patterns in *Physarum*. This demonstrates that $G_2$-phase–specific proteins are not among the main proteins of a cell.

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