ADP-ribosyltransferase in isolated nuclei during the cell cycle of *Physarum polycephalum*

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ADP-ribosyltransferase was measured in isolated nuclei of *Physarum polycephalum*. Activity was determined with and without exogenous DNA and histones. During the synchronous cell cycle the activity measured with exogenous substrates exhibited a typical peak enzyme pattern with a maximum of activity in S-phase, whereas activity measured without exogenous substrates displayed a step enzyme pattern. Both activities doubled in each cell cycle.

INTRODUCTION

ADP-ribosyltransferase (EC 2.4.2.30) catalyses the transfer of the ADP-ribose moiety of NAD⁺ to acceptor proteins (for review see Hayaishi & Ueda, 1982). The chromatin-associated enzyme is responsible for poly-(ADP-ribosyl)ation of several nuclear proteins, such as histones (Nishizuka *et al.*, 1968), high-mobility-group proteins (Tanuma *et al.*, 1985), a DNA topoisomerase (Ferro *et al.*, 1983), an endonuclease (Yoshihara *et al.*, 1974), RNA polymerase (Müller & Zahn, 1976), probably a DNA ligase (Creissen & Shall, 1982) and ADP-ribosyltransferase itself (Yoshihara *et al.*, 1977; Jump & Smulson, 1980; Ferro & Olivera, 1982; De Murcia *et al.*, 1983). This enzyme has been found in a variety of organisms, although the precise role of the enzyme and its product is not clearly understood at present. Involvement of poly(ADP-ribosyl)ation has been suggested in DNA repair, where poly(ADP-ribose) synthesis is stimulated after treatment of cells with DNA-damaging agents (Whish *et al.*, 1975; Smulson *et al.*, 1977; Durkacz *et al.*, 1980; Berger *et al.*, 1980), in DNA replication (Burzio & Koide, 1972), in RNA synthesis (Müller *et al.*, 1974), in differentiation and development (Rastl & Swetly, 1978; Isoai & Yasumasu, 1985) and in cell-cycle regulation (Kidwell & Mage, 1976). ADP-ribosyltransferase was investigated for fluctuations of activity during the cell cycle (Smulson *et al.*, 1971; Brightwell *et al.*, 1975; Kidwell & Mage, 1976; Tanuma *et al.*, 1978; Berger *et al.*, 1978a,b).

Brightwell *et al.* (1975) have studied ADP-ribosyltransferase in the myxomycete *Physarum polycephalum*. This organism offers the unique advantage of perfect natural synchrony of nuclear division. In a macroplasmidium containing up to 10⁹ nuclei mitosis occurs in 8–10 h intervals. The studies of ADP-ribosyltransferase by Brightwell *et al.* (1975) suggested a step pattern of the enzyme activity during the cell cycle of *Physarum*. Their investigation was performed in isolated nuclei with the use of an assay without exogenous substrates for poly(ADP-ribosyl)ation. Therefore they rather measured the availability of endogenous substrates than real ADP-ribosyltransferase activity. For this reason we measured ADP-ribosyltransferase activity in isolated nuclei in two ways: activity without exogenous substrates (termed 'endoADPRT' activity) and activity after addition of

exogenous histones and DNA (termed 'exoADPRT' activity). In fact we found completely different behaviour of each of these two enzyme activities during the cell cycle.

MATERIALS AND METHODS

Materials

[U-¹⁴C-adenine]NAD⁺ was obtained from Amersham International (Amersham, Bucks., U.K.). NAD⁺ and thymidine were purchased from Boehringer Mannheim (Mannheim, West Germany). Nicotinamide and dithiothreitol were from E. Merck (Darmstadt, West Germany). DNA (calf thymus, type I; highly polymerized), deoxyribonuclease I (bovine pancreas, type III), histone (calf thymus, type V-S) and theobromine were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.). All other reagents were of analytical grade.

Culture and techniques

All culture techniques were carried out under sterile conditions. Microplasmodia of *Physarum polycephalum* (strain M₃b, a Wis 1 isolate) were maintained in submersed shake culture in semi-defined medium (Daniel & Baldwin, 1964) supplemented with 0.013% haemoglobin instead of haematin.

Giant macroplasmodia were prepared by coalescence of microplasmodia (5 ml of plasmodial sediment) on filter paper supported by stainless-steel grids in plastic Bio-assay dishes as described by Affolter *et al.* (1979). The time of mitosis (telophase) was determined in ethanol-fixed smears under phase contrast (Guttes *et al.*, 1961). At selected time points explants (2.5 cm in diameter) were harvested. Nuclei were isolated and ADP-ribosyltransferase activity/nucleus was determined.

In order to convert enzyme activity/nucleus into enzyme activity/plasmodium, a separate macroplasmodium was inoculated and cultured in parallel under the same conditions. The total number of nuclei in this plasmodium was determined before the second mitosis. Under our experimental conditions we found 2 × 10⁸ nuclei/plasmodium. This value varied within ±10% between individual experiments. Because of the perfect natural synchrony of our system (all nuclei divide within

less than 5 min at telophase) we multiplied the counted number of nuclei by the factor 2 after the second mitosis and by the factor 4 after the third mitosis. The enzyme activity/nucleus was multiplied by the total number of nuclei/plasmodium.

Nuclear isolation was performed by the procedure of Mohberg & Rusch (1971). After dilution in incubation buffer (100 mM-Tris/HCl, pH 8.2, containing 10 mM-MgCl₂ and 1 mM-dithiothreitol) nuclei were counted in a haemocytometer.

ADP-ribosyltransferase assay

'EndoADPRT' assay. In accordance with Brightwell *et al.* (1975), the following assay was used. A batch of 4×10^6 nuclei was incubated at 4 °C in 500 µl of reaction mixture containing 100 mM-Tris/HCl, pH 8.2, 10 mM-MgCl₂, 1 mM-dithiothreitol, 60 mM-KCl, 4 mM-KF and 5 µM-[U-¹⁴C-adenine]NAD⁺ (40 µCi/µmol, 0.1 µCi/tube). The reaction was started by the addition of [¹⁴C]NAD⁺ and terminated after 0, 5 or 10 min with 250 µl of cold 60% (w/v) trichloroacetic acid. After 30 min at 0 °C the samples were collected on Whatman GF/C filters under suction. Filters were washed three times each with 3 ml of 10% (w/v) trichloroacetic acid and twice with 1 ml of ethanol each, dried and analysed for ¹⁴C radioactivity by liquid-scintillation counting.

'ExoADPRT' assay. A 10 µg portion of activated DNA, prepared by the method of Loeb (1969), and 10 µg of histone were added to the reaction mixture of the 'endoADPRT' assay. Samples were incubated for 0, 2.5 or 5 min.

Enzyme activities are expressed as amol of NAD⁺ incorporated into acid-insoluble material/min per nucleus.

RESULTS

Characterization of ADP-ribosyltransferase under various experimental conditions

ADP-ribosyltransferase activity was measured as described by Brightwell *et al.* (1975), with slight modifications (see the Materials and methods section). The enzyme activity measured under these assay conditions (without addition of exogenous substrates) is termed as 'endoADPRT' activity. The reaction exhibited nearly linear kinetics at 4 °C up to 15 min (Fig. 1). However, we measured enzyme activities (approx. 0.2 amol/min per nucleus), which were 100-fold higher than the values reported by Brightwell *et al.* (1975).

Because we assumed that under these conditions, with only endogenous substrates for poly(ADP-ribosylation), the maximum enzyme activity cannot be determined, we added exogenous histone and DNA to the assay system. The addition of either histone or DNA in each case showed a slight elevation of apparent enzyme activity (Fig. 1). Addition of both resulted in a more than 5-fold increase of enzyme activity; the maximum increase was achieved by addition of 10 µg of histone and 10 µg of DNA. The amount of exogenous histone is approximately equivalent to the amount of endogenous histone present in the assay system (4×10^6 nuclei). Since the 'endoADPRT' activity obviously does not resemble the true ADP-ribosyltransferase activity, we measured both enzyme activities during the cell cycle. The enzyme

Fig. 1. Time course of poly(ADP-ribose) synthesis in isolated nuclei of *Physarum polycephalum* microplasmodia

The reaction mixtures for determination of 'endoADPRT' activity (○) and 'exoADPRT' activity (●) are described in the Materials and methods section. The reactions were carried out at 4 °C for various times. Enzyme activities were expressed as amol of NAD⁺ incorporated into acid-insoluble material/nucleus. In parallel, 500 µM-nicotinamide (■) was added to reaction mixtures after 10 min incubation (indicated by arrows). In addition, 'endoADPRT' activity was measured in the presence of 500 µM-nicotinamide (■), 10 µg of histone (△) or 10 µg of DNA (□).

activity measured after addition of exogenous histone and DNA is termed 'exoADPRT' activity. The kinetics of 'exoADPRT' activity were linear only for short incubation times. The rate of incorporation decreased during the course of the enzyme reaction (Fig. 1).

The reaction temperature of 4 °C was chosen since lowering of the reaction temperature from 20 °C to 4 °C showed a 3-fold elevation of the enzyme activities. This is in accordance with the observation by Brightwell *et al.* (1975) that enzyme activity continuously increased when the incubation temperature was lowered from 35 °C to 10 °C; the range below 10 °C has not been investigated by these authors.

Enzyme activity was inhibited by nicotinamide (Fig. 1); the same effect (results not shown) was observed with thymidine and theobromine, which are known to be inhibitors of ADP-ribosyltransferase (Preiss *et al.*, 1971; Shall *et al.*, 1977).

To exclude the possibility that the poly(ADP-ribosylation) is accompanied by a degradation of the reaction product, we added nicotinamide after 10 min of incubation to the reaction assay. As shown in Fig. 1, the reaction is stopped and no degradation of the synthesized poly(ADP-ribose) was observed.

Fig. 2. ADP-ribosyltransferase activities during the synchronous nuclear division cycle

A giant macroplasmidium was prepared as described in the Materials and methods section. At selected time points (starting 1 h before the second mitosis) pieces (2.5 cm in diameter) were explanted off the plasmodium and harvested. Isolated nuclei were analysed for 'endoADPRT' (○) and 'exoADPRT' (●) activities as described in the Materials and methods section. Incubation times were 0, 5 and 10 min for 'endoADPRT' and 0, 2.5 and 5 min for 'exoADPRT'. The enzyme activities were expressed as amol of NAD^+ incorporated into acid-insoluble material/min per nucleus (a). The ADP-ribosyltransferase activities of the whole macroplasmidium were calculated as nmol of NAD^+ /min per macroplasmidium (b). The approximate duration of the cell cycle phases is indicated. M2 and M3 indicate the times of the second and third mitoses after inoculation of the macroplasmidium.

ADP-ribosyltransferase during the synchronous nuclear division cycle

After we had established assay conditions for measuring enzyme activities, we investigated 'endoADPRT' and 'exoADPRT' activities during the nuclear division cycle, as previously done by Brightwell *et al.* (1975) for 'endoADPRT'. The 'endoADPRT' activity followed the same pattern as already described, with a minimum after mitosis, a subsequent increase during S-phase (*Physarum* plasmodia lack a G_1 -period) and a plateau during the rest of the cycle. In contrast, 'exoADPRT' activity exhibited a pronounced maximum of activity in

mid-S-phase (Fig. 2a). The usual presentation of enzyme activity/nucleus as in Fig. 2(a) causes a sharp decline after nuclear division owing to the distribution of the existing enzyme activity to the double number of nuclei. In order to demonstrate the real course of enzyme activity during progression through the cell cycle, we plotted the same data as enzyme activities/cell, as described in the Materials and methods section. This plot (Fig. 2b) revealed a typical peak enzyme pattern of ADP-ribosyltransferase measured as 'exoADPRT'. The increase of enzyme activity was already induced before nuclear division, reaching a distinct maximum in mid-S-phase and decreasing continuously until late G_2 -period. The enzyme activity was doubled during each cell cycle. The 'endoADPRT' activity increased considerably later (0.5 h after mitosis), reaching a plateau during late S-phase.

DISCUSSION

We have conclusively demonstrated that poly(ADP-ribosylation) must be divided into two distinct activities: (1) 'exoADPRT' activity, which represents the maximum activity measured under conditions of substrate saturation; (2) 'endoADPRT' activity, which on the other hand reflects the physiological availability of acceptor proteins for poly(ADP-ribosylation). The latter reaction has already been measured during the cell cycle by Brightwell *et al.* (1975); our results are in agreement with that report.

ADP-ribosyltransferase exhibits a typical peak enzyme pattern during the cell cycle of *Physarum*, with a maximum of activity in mid-S-phase. The increase of enzyme activity starts before mitosis (mitosis precedes S-phase without an intermitting G_1 -period). The enzyme activity is doubled in each cell cycle. These features are common to some other enzymes involved in DNA replication that have been studied during the cell cycle of *Physarum*, e.g. DNA polymerase (Brewer & Rusch, 1966), thymidine kinase and thymidylate synthetase (Gröbner & Loidl, 1982). Interestingly, NAD pyrophosphorylase, the enzyme responsible for the biosynthesis of NAD^+ , the substrate for ADP-ribosyltransferase, exhibits a congruent peak of activity in mid-S-phase (Solao & Shall, 1971). In all cases enzyme activities began to increase before the start of mitosis, which is presumably due to the lack of a G_1 -period. Since ADP-ribosyltransferase is induced at the same time as several investigated enzymes that are necessary for DNA synthesis, it is probable that poly(ADP-ribosylation) plays a role during DNA replication. It is suggestive that the induction of these enzymes is under a common regulatory control.

Changes in the activity of ADP-ribosyltransferase during the cell cycle have already been studied during the induction-synchronized cell cycle of HeLa cells (Smulson *et al.*, 1971; Kidwell & Mage, 1976; Tanuma *et al.*, 1978). The common result of these studies is that poly(ADP-ribose)synthesis shows a peak. However, the results differ considerably with respect to the location of this peak in the cell cycle, varying from the G_1 -period until the G_2 -period. Obviously these conflicting results have been obtained under different experimental conditions. It is quite clear that *Physarum* is a most suitable system for study of the cell-cycle-dependence of ADP-ribosyltransferase owing to its natural mitotic synchrony. The present work conclusively shows that the peak of enzyme activity is located in S-phase.

It remains to be explained why 'endoADPRT' displays a different activity pattern from 'exoADPRT' activity. 'EndoADPRT' activity shows a delayed increase (0.5 h after mitosis) as compared with 'exoADPRT' activity (0.5 h before mitosis). The doubling of 'endoADPRT' activity takes place at a time when histone biosynthesis occurs (Schofield & Walker, 1982). Since histones are known to be a substrate for ADP-ribosyltransferase (Burzio & Koide, 1972; Tanuma *et al.*, 1977; Riquelme *et al.*, 1979), the increase of the 'endoADPRT' activity could reflect the poly(ADP-ribosyl)ation of the newly synthesized histones. The plateau of 'endoADPRT' activity could be partially due to a continuous turnover of poly(ADP-ribose). Another possible explanation is that the plateau of 'endoADPRT' activity is caused by a continuous automodification of the enzyme itself, as already known from other systems (Yoshihara *et al.*, 1977; Jump & Smulson, 1980; Ferro & Olivera, 1982; De Murcia *et al.*, 1983).

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