Molecular constituents of the replication apparatus in the plasmodium of Physarum polycephalum: identification by photoaffinity labelling

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The plasmodium of Physarum polycephalum has long been considered a model system for syncytically growing cells, but important details of the DNA replication apparatus, such as the DNA polymerase ε and other replication factors, have not been detected. In this study, a new variation of photoaffinity labelling and immunoblotting was used to detect DNA polymerases and other factors in nuclear extracts of P. polycephalum. Proteins were specifically cross-linked with photoreactive arylazido-dCMP residues incorporated during extension of template-primer DNA. The DNA synthesized in situ was labelled. After nucleolytic removal of protruding DNA, the proteins were separated by SDS-gel electrophoresis, electroblotted on membranes and subjected to autoradiography. The α, δ, ε and β-like DNA polymerases were labelled, as were histones and replication-factor-like proteins. Cytoplasmic extracts were devoid of these species. Abundant proliferating-cell nuclear antigen and replication protein A large subunit were labelled and found to be of unusual mass. A number of subunits of purified DNA polymerase holoenzymes were labelled. In contrast, only the DNA-polymerizing subunits could be labelled in nuclear extracts. Higher-order complexes in the nuclear extract may make subunits inaccessible to photo-cross-linking. Complex formation is promoted by β-poly(L-malate), a plasmodium-specific putative storage and carrier molecule that supports DNA replication in the synchronized nuclei. Percoll, a polyvinylpyrrolidone-coated colloidal silica, partially disrupted these complexes. A 200 kDa fragment of DNA polymerase ε and a 135 kDa β-like DNA polymerase did not participate in the complexes, suggesting functions unlike those of the other polymerases. DNA polymerase molecules were intact during proliferation of plasmodia, but were nicked before their clearance from the nuclei at growth arrest.

Keywords: DNA replication, syncytium, protist, photoaffinity labelling, cross-linking

INTRODUCTION

Eukaryotic nucleic acid replication has been intensively investigated in a series of model systems such as yeast, mouse and human cells, and it has been assumed that the constituents and mechanisms of the replicating systems are largely conserved among eukaryons, and also that they share functional similarities with those of bacteria and probably archaea (for a recent review see Hindges & Hubscher, 1997). It is not clear what variations might...
exist for organisms which emerged early in evolution or have an unusual cellular organization. *Physarum polycephalum* meets both these criteria. Previous results placed this slime mould among protists (Margulis & Schwartz, 1982; Johansen *et al.*, 1988; Cavalier-Smith, 1993; Woese, 1994), while recent findings suggested its evolution among plants, fungi and animals (Baldauf & Doolittle, 1997).

Nuclei of plasmodial *P. polycephalum* divide with high synchrony without passing through cytokinesis, giving rise to a large multinuclear syncytium (for a review see Burland *et al.*, 1993). To gain insight into evolutionary variability and synchrony maintenance, we began to characterize the replication apparatus of *P. polycephalum*. DNA polymerase α-primase complex (140 kDa polymerizing subunit, two primase subunits of 53 and 58 kDa, and a 82 kDa polypeptide) (Weber *et al.*, 1988; Achhammer *et al.*, 1992; Angerer & Holler, 1995), DNA polymerase δ (125 kDa) and PCNA (35 kDa) (Achhammer *et al.*, 1995) have been identified and characterized. The DNA polymerase α 140 kDa subunit was proteolytically unstable, forming an inactive 110 kDa fragment (Weber *et al.*, 1988). The polymerase was found to differ in several respects from polymerases of yeasts and higher eukaryotes (Achhammer *et al.*, 1995).

In search of a molecular device that could account for the synchrony of nuclear division, we have identified an unusual polymer, β-poly(-l-malate) (PMLA) (Fischer *et al.*, 1989). This polyanion is specifically synthesized in plasmodia but not in uninuclear cell forms of *P. polycephalum*. Its mode of action has been tentatively attributed to transport and storage of histones, DNA polymerases α and δ, and other nuclear proteins in DNA replication (Fischer *et al.*, 1989; Holler *et al.*, 1992b; Achhammer *et al.*, 1995; Angerer & Holler, 1995). β-like DNA polymerase, which is probably not active in DNA replication, does not bind to PMLA. In the complexes, the activities of DNA polymerases are reversibly inhibited. The reaction is highly myxomycete-specific since DNA polymerases of other eukaryotes or bacteria are not inhibited (Fischer *et al.*, 1989; Holler *et al.*, 1992b, and unpublished data).

Our investigation was intended to gain further insight into the replication apparatus of *P. polycephalum* and to compare it with the systems of higher eukaryotes. We wanted to validate and expand the knowledge on the role of PMLA. We concentrated on identifying the still missing main players DNA polymerase ε and replication factors. A suitable method for recognizing such proteins was the photoaffinity technique designed to label enzymically active DNA polymerases (Doronin *et al.*, 1992; Lavrik & Potapova, 1994; Safronov *et al.*, 1997) and replication protein A (RP-A) (Lavrik *et al.*, 1998) in purified systems. By introducing highly efficient photo-cross-linkers and an optimized protocol, labelling was performed in cell extracts. [α-32P]dNTP and a photo-reactive arylazido derivative of dCTP were incorporated into nuclease-activated salmon testis DNA simultaneously. The nascent DNA was cross-linked by irradiation to DNA-bound proteins. After SDS-PAGE and electroblotting, DNA polymerase α, δ, ε, PCNA, RP-A and replication factor C (RF-C) proteins were identified by autoradiography and immunostaining. The technique allowed us to purify DNA polymerase ε and to observe effects with PMLA and oligophosphate diphosphonucleotides. The function of PMLA was evident from the results of labelling. It integrated DNA polymerases into higher-order complexes. At the beginning of the starvation-induced switch to the non-replicative phase of the life cycle, the polymerase components of the complexes were proteolytically nicked before they disappeared from the nuclei.

**METHODS**

**Materials.** Benzaldehyde grade II (25 units g⁻¹) was purchased from Merck. [α-32P]dNTPs (3000 Ci mmol⁻¹, 1 Ci = 3.7 × 10¹⁰ Bq) and molecular mass markers were purchased from Amersham. Taq polynucleotide kinase was purchased from Life Technologies. DNA polymerase I from *Escherichia coli* was purchased from Boehringer Mannheim. Purified *P. polycephalum* DNA polymerase α was the phosphocellulose fraction according to Weber *et al.* (1988) and Achhammer *et al.* (1992). *P. polycephalum* DNA polymerase δ was purified as described by Achhammer *et al.* (1995). The enzymes were stored at −70 °C in the presence of 50% (v/v) glycerol, 50 mM Tris/HCl pH 7-5, 1 mM EDTA and 1 mM 2-mercaptoethanol. All other reagents were of analytical grade and were purchased from Merck. Rabbit antisera against *P. polycephalum* DNA polymerases α and ε (both as holoenzymes) and rabbit antisera against peptides of *P. polycephalum* DNA polymerase δ have been described (Achhammer *et al.*, 1992, 1995). Chicken antisera against human PCNA was a gift from Dr B. Stillman (Cold Spring Harbor, USA). Rabbit antisera against human RP-A, 70 kDa subunit, was a gift from Dr K. Weisshart (Jena, Germany). Antiserum against yeast replication factor C4 (RF-C4) was a gift from Dr P. M. J. Burgers (St Louis, USA). Peroxidase-coupled anti-rabbit IgG and anti-chicken IgY antibodies were purchased from Pierce.

**Photoactive analogues of nucleotide triphosphates.** The photoactive nucleotides exo-N-[2-(5-azo-2-nitrobenzen- amidino)ethyl]deoxyoctydine-5'-triphosphate (ANBdCTP) and exo-N-[[(4-azido-2,3,5,6-tetrafluorobenzylidene)hydrazino] carbonyl]butyl]carbonyl deoxyoctydine-5'-triphosphate (AF-BdCTP) were synthesized as described previously (Safronov *et al.*, 1997). Their structures are shown in Fig. 1. Absorbance maxima were at 303 nm (9000 M⁻¹ cm⁻¹) for AFdCTP and at 320 nm (8000 M⁻¹ cm⁻¹) for ANBdCTP. A cut-off filter (06 absorbance units at 310 nm and complete cut-off at 295 nm) was used during photo-cross-linking to protect the samples against far-UV light. The light source was an Osram XBO 150 W (7-5 A) high-pressure xenon lamp. The distance between the source and the sample was 15 cm. The nucleotide analogues were photo-inactivated with half-lives of 1 min (AFBdCTP) and 3 min (ANBdCTP); they were superior to previously synthesized reagents and to commercially available nucleotides such as BrdUTP in their high cross-linking yields (5–7% of the photoactive DNA cross-linked with bound proteins) and their rapid photoactivation (Safronov *et al.*, 1997).

**Template-primers.** Activated salmon testis DNA was prepared
Fig. 1. Structures of AFBdCTP and ANBdCTP.

According to Holler et al. (1987). Synthetic 16-mer primer 5'-GTTTTTCAGTCAGCA 3' was annealed to a synthetic 26-mer template 3'-AAAAAGGTACAGTCGGTTACCTAC 5' or to M13mp18 DNA 3'-CAAAAGGTACAGTCGGTTACCTAC 5'. The primer was 5'-end labelled with [32P]ATP as described by Sambrook et al. (1989), and was separated from excess reactants on NENSORB-20 (DuPont). Other synthetic template-primers were poly(dA)/poly(dT) 12-18 (10/1), poly(dA)/poly(dT) 12-18 (10/1) and poly(dT)/poly(dA) 10-16 (20/1), prepared according to Achhammer et al. (1995).

DNA polymerases. DNA polymerase ε was separated from the other DNA polymerases on phosphocellulose (Achhammer et al., 1995); it eluted with 410 mM KCl close to the position for DNA polymerase α (590 mM KCl). Purification was continued over hydroxyapatite (elution with 180-200 mM KCl), ssDNA cellulose (340 mM KCl), and MonoQ (270 mM KCl). At all stages of the purification the polymerase was identified by photolabelling and its molecular mass by 230 kDa. After chromatography on MonoQ, the preparation had high specific activity but contained mainly proteolytically nicked polymerase and AFBdCTP were irradiated for 2 min; this polymerase-catalysed ligation of the cross-linked dCTP analogue with high and low molecular mass marker proteins from Sigma.

Labelling of DNA polymerases in nuclear extracts

Labelling of DNA polymerases and DNA-interacting proteins. AFBdCTP was used in most of the experiments because it had a higher labelling efficiency than ANBdCTP. Samples were labelled in a solution (20 μl) containing 0.05-0.5 μM DNA polymerase, 3-5 μM [α-32P]dATP (3-300 Ci mmol-1), 50-100 μM AFBdCTP, 50 mM MOPS buffer (pH 7.5), 50 mM MgCl₂, 3 mM EDTA and occasionally 33 μM each of dGTP and dTTP. The activities of DNA polymerases and DNA-interacting proteins were routinely standardized by assaying acid-extractable radioactivity incorporated into DNA synthesized from [α-32P]dCTP as a substrate. For DNA polymerases α, ε, and M, activated salmon testis DNA was used as template-primer (Holler et al., 1987), and for DNA polymerases δ and ε, poly(dA)/poly(dT) 12-18 (Achhammer et al., 1995). A single unit of enzyme activity represents the incorporation of 1 nmol bases during 1 h at 37 °C in the standard assay.
coli DNA polymerase I in the photoaffinity reaction mixture as a positive control and omitted the photoreactive nucleotide or irradiation as a negative control.

Primer extension with AFbdCTP or ANBdCTP was carried out in 4.5–10 μl reaction mixture containing 0.005–0.02 units of highly purified *P. polycephalum* DNA polymerase α, 0.01–0.1 μM synthetic 5’-[32P]primer and template (synthetic 26-mer or M13mp18 DNA), indicated concentrations of photoreactive nucleotide, 50 mM Tris/HCl pH 7.5, 50 mM KCl, 10 mM MgCl2, 0.3 mM EDTA and 0.01 mM 2-dithioerythritol. Reaction was allowed for 15 min at 37 °C. In some assays natural dNTPs were added, and the incubation was continued for 15 min. To terminate the reaction, 5 μl stop solution [80% (v/v) aqueous formamide, 50 mM Tris/boric acid buffer pH 8.3, 1 mM EDTA and 0.1% bromophenol blue] was added. Samples (5–10 μl) were analysed on 20% polyacrylamide-urea gels (Sambrook *et al.*, 1989).

RESULTS AND DISCUSSION

Incorporation of photoreactive nucleotides into DNA during primer extension

Incorporating the dCTP analogues into DNA during primer extension catalysed by *P. polycephalum* DNA polymerase α was confirmed to give a single product at low concentrations of AFBdCTP (Fig. 2a, lane 4, band d). Incorporation was inhibited when dCTP was included in the polymerization mixture (Fig. 2a, lanes 5–8), resulting in the extension of primer (band a) by a single dCMP (band b). The inhibition was complete for 50 μM dCTP (Fig. 2a, lanes 6 and 8). This suggested that the dCTP derivatives represented functional analogues of dCTP. The electrophoretic mobility of the product d in Fig. 2(a) was less than that of products having dCMP (Fig. 2a, band b) or dCMP together with dAMP incorporated (Fig. 2a, band c) and was in agreement with the bulkiness of the photoreactive nucleotide. An additional product, band e, was seen at elevated concentrations of AFBdCTP after 1 min (Fig. 2a, lane 5) and especially after prolonged polymerization (Fig. 2b). Band e represents an incorporation of AFBdCTP opposite to dT and next to dG of the 26-mer template. Band f in Fig. 2(c), lane 2, represents an incorporation opposite to dC of the M13mp18 template. Other results indicated an incorporation in the position of dT. Primer extension with ANBdCTP showed a comparably higher degree of fidelity (Fig. 2c, lanes 1 and 3), corresponding to a single product in band g. Misincorporation became complicated when dGTP or dATP was also included into the polymerization mixture, as shown by the emergence of several products in Fig. 2(c), lanes 3 and 4, and Fig. 2(d), lanes 2 and 3. The observed low level of fidelity reflected the general tendency of a polymerization catalysed by DNA polymerase α. Full-length primer extension was probed in the presence of nucleotides dATP, dGTP, dTTP and the photoreactive dCTP analogue. Products h and i in Fig. 2(e), lanes 3 and 4, migrated close to product k in the presence of the four natural dNTPs (Fig. 2e, lane 5), suggesting a nearly full-length polymerization. In summary, the results indicated the incorporation of AFBdCTP (and also ANBdCTP) by *P. polycephalum* DNA polymerase α preferentially in the dC position and with lower frequencies in the dT, dA and dG positions.

![Fig. 2. Incorporation of photoreactive nucleotides during primer extension. Denaturing urea gels are shown for products obtained by extension of synthetic 5’-[32P]16-mer primer/26-mer template (0.1 μM) with various nucleotides. The reaction was catalysed by 0.015 units DNA polymerase α. (a) Demonstration of competition between AFbdCTP and dCTP. (b) Time dependence of incorporation of AFbdCTP (50 μM). (c) Effect of added dGTP on primer extension; M13mp18 DNA was the template. (d) Effect of added dATP on primer extension; synthetic 26 mer-DNA was the template. (e) Extension of 5’-[32P]16-mer primer/26-mer template with ANBdCTP or AFbdCTP in the presence of complementary dNTPs.](image-url)
Labelling of DNA polymerases in nuclear extracts

**Fig. 3.** Photoaffinity labelling of purified DNA polymerases from *P. polycephalum*. The standard labelling conditions were used, with 0.2 μM synthetic 26-mer template/16-mer primer, 50 μM AFBdCTP, 3 μM [α-32P]dATP (3 CI mmol⁻¹), 0.1–0.3 units DNA polymerase, and autoradiography from membranes after electroblotting (see also Methods). ‘-UV’ denotes controls without irradiation. (a) Lanes 1 and 2, 0.12 units DNA polymerase; digestion with nuclease omitted. Lanes 3 and 4, the same but 0.24 units DNA polymerase. Lanes 5 and 6, same as in lanes 3 and 4 but using the catalytically competent condition employing the 26-mer template/5'-[α-32P]16-mer primer. DNA was not digested. To test for primer extension, unlabelled 26-mer template/16-mer primer was cross-linked to polymerase first, and [α-32P]dATP was then added (lanes 7 and 8). (b) DNA polymerase δ. (c) DNA digestion by benzonase following photolabelling. Lanes 1–3, synthetic template-primer in the absence (lane 2) and the presence (lane 3) of benzonase; lanes 4–8, activated salmon testis DNA in the absence (lanes 4 and 5) and in the presence (lanes 6–8) of benzonase; lanes 6–8, digestion for 5, 10 and 30 min at 37 °C. (d) Labelling in the presence of 3, 6, 9 and 12 μg activated DNA. The reaction mixture also contained dGTP and dTTP. DNA was digested after photo-cross-linking. (e) Comparison of synthetic template-primer with activated DNA and of [α-32P]dATP with [α-32P]dTTP. Reaction mixtures contained 100 μM AFBdCTP. DNA was digested after UV irradiation. Lanes 1–3, 2.8 μM [α-32P]dTTP (50 CI mmol⁻¹); dATP, dGTP, dTTP; lanes 4 and 5, 2.8 μM [α-32P]dATP (50 CI mmol⁻¹); dGTP, dTTP; lanes 1 and 4, synthetic 26-mer template/16-mer primer; lanes 2, 3 and 5, activated DNA.

**Labelling of purified DNA polymerases from *P. polycephalum***

Photoaffinity labelling was first applied to purified DNA polymerases to test its efficacy and to optimize the assay conditions. The method was then intended to be used to identify and purify *P. polycephalum* DNA polymerase ε. The final goal was to characterize *P. polycephalum* cellular extracts. To optimize the cross-linking conditions, the synthetic template-primer originally used was replaced by the less expensive and abundantly available DNase-I-activated salmon testis DNA. Excess DNA was removed by digestion with the nuclease benzonase. After electrophoretic separation, the labelled proteins were concentrated by electroblotting onto the surface of a membrane. The time for autoradiography was extended from 12 h to 6–8 d.

The intensities after improvement of the method are shown in Fig. 3 for purified *P. polycephalum* DNA polymerases. The effect of DNA digestion is noted as an increase in electrophoretic mobility for the synthetic template-primer in Fig. 3(c), lanes 2 and 3, and as a resolution into bands for activated salmon testis DNA in Fig. 3(c), lanes 5 and 6. The labelled oligonucleotide tag, which remained attached after digestion, tended to
increase the molecular mass in comparison with the non-labelled proteins. We found that the increase was reproducible (Fig. 3c, lanes 6–8) and was an intrinsic property of each protein. It was 5–15 kDa for DNA polymerases and their subunits. For replication factors and histones (see below) an increase was almost undetectable. Among the various types of template-primers examined [including poly(rA)/p(dT)\textsubscript{12-18}, poly[\textup{dA}]\textsubscript{16}/p[\textup{dT}]\textsubscript{12-18} (10/1), poly[\textup{dT}]\textsubscript{12-18}/p[\textup{rA}]\textsubscript{16} (20/1), results not shown], activated DNA and the synthetic template-primer were optimal and gave comparable results (Fig. 3c, lanes 3–8). In the absence of UV irradiation, labelling was not observed; in cases, such as that in Fig. 3(a) lane 7, samples were probably insufficiently protected against light.

The bands in Fig. 3 (except panel b) were reconcilable with the reported molecular masses of subunits of the DNA polymerase \(\alpha\)-primase complex. The subunits are listed on the right side of Fig. 3(a) and refer to the 140 kDa DNA polymerizing protein, the 82 kDa interconnecting protein, and the 58 kDa/53 kDa primase subunits (Weber et al., 1988; Achhammer et al., 1992; Angerer & Holler, 1995). The band at 82 kDa was only stained in the presence of the synthetic template-primer (arrows in Fig. 3). It is not clear why it was not seen in the presence of activated DNA (Fig. 3c, lanes 6–8, and Fig. 3d). Other labelled bands, especially those at 100 and 110 kDa in Fig. 3(a), lane 2, Fig. 3(c), lanes 6–8, and Fig. 3(e), lanes 4 and 5, were ascribed to proteolytic degradation products of DNA polymerase \(\alpha\) (Weber et al., 1988). For DNA polymerase \(\delta\) in Fig. 3(b), bands at 140 and 45 kDa were identified. They could be attributed to the polymerizing (125 kDa) subunit and to an additional subunit (approximately 40 kDa) of unknown function by relating them to the existence of subunits in mammalian or Saccharomyces cerevisiae DNA polymerase \(\delta\) (125 kDa polymerizing subunit and 50–53 kDa subunits of unknown functions: Zhang et al., 1995; Sugimoto et al., 1995).

The bands at 230 and 68 kDa could not be reconciled with known \textit{P. polycephalum} DNA polymerases or their degradation products. The possibility that the bands at 68 and 230 kDa in Fig. 3(a, c–e) resulted from non-specific labelling was first considered. However, their intensities depended on the protein concentration (Fig. 3a, lanes 2 and 4). A dependence on the concentration of activated DNA is seen for the band at 140 kDa (the DNA-polymerizing subunit) but not for the bands at 68 and 230 kDa in Fig. 3(d). This seemed to indicate different DNA binding affinities. Addition of dGTP and dTTP, each at 33 \(\mu\)M, substantially increased the band intensities (data not shown). The presence of dCTP (500 \(\mu\)M) in large excess over AFBdCTP (50 \(\mu\)M) inhibited labelling (data not shown), in agreement with the competitive status of these nucleotides. The 110 and 140 kDa proteins were labelled when \([\alpha-32P]\)dATP (Fig.
Fig. 5. Photoaffinity labelling of spontaneously degrading purified DNA polymerase \( \varepsilon \). Samples of DNA polymerase \( \varepsilon \) kept at \(-20^\circ C\) were analysed at the indicated times. Standard labelling conditions included activated DNA, 50 \( \mu \)M AFBdCTP, dGTP and dTTP (33 \( \mu \)M each), 0.1 \( \mu \)M \([\alpha-32P]dTTP\) (3000 Ci mmol\(^{-1}\)) and digestion with benzonase. Polymerase activity according to the standard assay was 0.5 units at day zero and 0.4 units after 140 d. The results for 75 and 140 d are shown in duplicate.

Identification of DNA polymerase \( \varepsilon \)

Proteins identified by the labelled bands at 68 and 230 kDa were purified from plasmodial extracts employing the photoaffinity labelling assay. The purified preparation confirmed the labelling (Fig. 4c, arrows in lanes 1 and 3). The intensity depended on the concentrations of protein (Fig. 4a), AFBdCTP (Fig. 4b), and activated DNA (Fig. 4c). The purified preparation was able to support DNA synthesis, preferring poly(dA)/poly(dT) as template-primer and had an intrinsic 3'-5' exonuclease proofreading activity (data not shown). Activity was inhibited by N-ethylmaleimide (70% inhibition at 1.2 mM), aphidicolin (50% inhibition at 5 \( \mu \)g ml\(^{-1}\)), dimethylsulfoxide (50% inhibition at 10% aqueous dimethylsulfoxide), butylphenyl-dGTP (83% at 10 \( \mu \)M) and heparin (30% at 0.22 \( \mu \)g ml\(^{-1}\)). The results fitted the published criteria for type \( \varepsilon \) DNA polymerase, the protein at 230 kDa representing the polymerizing subunit, and the protein at 68 kDa, a subunit of unknown function. Corresponding sizes have been reported for DNA polymerase \( \varepsilon \) of \( S.\) \textit{cerevisiae} (for a recent review see Sugino, 1995) and higher eukaryotes (Chui & Linn, 1995; for a recent
The e-type of polymerase is known to synthesize DNA with higher processivity and fidelity than the α-type, in agreement with the labelling patterns noticed in Fig. 3(e). When tested for its sensitivity towards PMLA, *P. polypephalum* DNA polymerase e was inhibited to 50% in the presence of 0.4 μg polymer ml⁻¹, in agreement with the findings for DNA polymerase α and δ of *P. polypephalum* (Fischer et al., 1989; Achhammer et al., 1995).

**Labelling of nicked DNA polymerases**

In Fig. 3(a, c, e), 100 and 110 kDa labelled proteins were fragments of DNA polymerase α. In Fig. 5, on storage of DNA polymerase ε at −20 °C, photolabelled polypeptides decreased in size dramatically (from 230 to 30–70 kDa). This was accompanied by a 40% decrease in activity by the standard polymerase assay. Thus even extensive proteolytic nicking does not necessarily abolish polymerase activity for affinity labelling. DNA polymerase ε has been reported to be particularly sensitive to proteolysis in preparations from several organisms (Kesti & Syvaoja, 1991; Uitto et al., 1995).

**Photoaffinity labelling of cellular extracts**

In investigating the cytoplasmic and nuclear extracts of plasmodia we asked the following questions. (i) Is the technique sensitive enough to detect DNA polymerases in plasmodial extracts? (ii) Are DNA polymerases and replication factors restricted to nuclei? (iii) Are DNA polymerases and subunits labelled to equal intensities in purified preparations and extracts? (iv) Are the equivalents of PCNA, RP-A and RF-C labelled, and how do their molecular masses compare with those from yeast and higher eukaryotes? (v) Is labelling affected by PMLA? (vi) Does the pattern of labelled proteins change during (natural) growth arrest?

A variety of proteins were labelled in the nuclear extract (Figs 6–8). Electrobloctting improved their detection (Fig. 6b, lanes 1–6). Band patterns were similar for AFBdCTP in (Fig. 6a, lane 5) and ANBdCTP in (Fig. 6a, lane 7), albeit of higher intensity for the former. DNA polymerases α and ε were among the labelled proteins identified in Fig. 6(b), lanes 1 and 2. The immunostained bands matched the subunits. Antiserum against DNA polymerase δ-peptide stained a band of 125 kDa in Fig. 6(b), lane 3. Taking into account 15 kDa of the [32P]DNA-tag after labelling, the band would be shifted into the 140 kDa position. This was the region of bands for labelled type-α and β-like DNA polymerases (135 kDa for unlabelled β-like DNA polymerase; Holler et al., 1987). The proximity of several bands was indicated by the relatively broad labelling in the 135–150 kDa region (for instance in Fig. 6b, lanes 2, 6 and 8, and in Fig. 7a, lanes 1 and 3).

Antiserum against human PCNA heavily stained a protein at 46 kDa (Fig. 6b, lane 5) that was also intensely labelled (Fig. 6b, lane 6). In the DNA metabolism of eukaryotes PCNA is an indispensable factor that, among several other functions, enhances the processivity of DNA synthesis by DNA polymerase δ. It is a highly conserved 37 kDa polypeptide that oligomerizes to form a doughnut-like DNA clamp (for a recent review see Hübscher et al., 1996). A 46 kDa PCNA analogue would thus be unusual. Its heavy immunostaining and photo-hybridisation indicate that the 46 kDa PCNA-like species was very abundant and probably significant. We have recently purified and characterized a 35 kDa PCNA in *P. polypephalum* (Achhammer et al., 1995). It is now assumed that this form of PCNA is the product of partial proteolysis. The 46 kDa PCNA is similar in mass to the labelled 40 kDa subunit of DNA polymerase δ seen in Fig. 3(b). It presumably also functions as a subunit of DNA polymerase δ.

Staining with antiserum against the 70 kDa subunit of human RP-A indicated bands at 54 kDa (intense) and 62 kDa (weak) (Fig. 6b, lane 7). Intense labelling could be observed for the 54 kDa band (Fig. 6b, lane 8). Labelling of the 70 kDa subunit of human RP-A has been reported recently, demonstrating accessibility of the factor to photoreactive DNA (Lavrik et al., 1998). RP-A is a heterotrimeric, single-stranded DNA-binding protein required for eukaryotic DNA metabolism (for a recent review see Wold, 1997). The molecular mass of the large *P. polypephalum* RP-A subunit could be reconciled with the size (53 kDa) of a proteolytic fragment derived from the bovine 70 kDa RP-A protein (Nasheuer et al., 1992).

Antiserum against yeast RF-C subunit C4 stained bands at 31, 38 (barely visible), and 72 kDa (Fig. 6c, lane 1). Marginal labelling was also observed at 31 and 36 kDa, and no labelling at 72 kDa (arrows in Fig. 6c, lanes 2 and 3). In yeast and higher eukaryotes, RF-C is a complex of polypeptides with molecular masses of approximately 140, 40, 38, 37 and 36 kDa. It is an essential component of the replication machinery of eukaryotes, functioning in the loading of the PCNA clamp to DNA (for a recent review see Hubscher et al., 1996). The indication of three bands for RF-C in Fig. 6(c) is in agreement with the cross-reactivity of the antibody to the three small subunits of RF-C from *S. cerevisiae* (Gary & Burgers, 1995). The low level of photoaffinity labelling is reconciled with the low abundance of this factor.

The identity and functions of DNA polymerases were supported by the responses to specific inhibitors of DNA synthesis. Aphidicolin, a known inhibitor of DNA polymerases α, δ and ε (Weber et al., 1988; Angerer & Holler, 1995; Achhammer et al., 1995; this work), inhibited labelling of bands in positions 140–230 kDa, in agreement with the presumed types of polymerases banding in these positions (arrows in Fig. 7b, lanes 3–6). Dinucleotide oligophosphates AP₃ and AP₄, which are contained in plasmodia of *P. polypephalum* (Garrison & Barnes, 1992), are considered to be alarmones that arrest growth in response to environmental stress (for a review see Remy, 1992). These
Fig. 6. Western blots and photoaffinity labelling of DNA polymerases and factors in nuclear extracts. Extracts were prepared from nuclei of 2- and 3-d-old microplasmodia by centrifugation over a Percoll density gradient unless stated otherwise. The standard assay was used for labelling. (a) Increase in resolution and intensity by electroblotting before autoradiography; 3-d-old plasmodia were compared for AFBDCTP or ANBdCTP in the reaction mixture. Lanes 1–4, autoradiography of a dried SDS-polyacrylamide (75% acrylamide) gel. Lanes 5–8, autoradiography of the membrane after electroblotting; lanes 1 and 5, 100 mM AFBDCTP (2 min UV irradiation); lanes 3 and 7, 100 mM ANBdCTP (2 min UV irradiation); lanes 2, 4, 6 and 8 are controls in the absence of irradiation. Positions of bands are marked by dots in lane 5. (b) Comparison of band patterns obtained by Western blotting and photoaffinity labelling. Lane 1, Western blot (ECL staining) with antisera against DNA polymerases \( \alpha \) and \( \epsilon \) for 2-3-d-old plasmodia; lane 2, photoaffinity labelling for 2-d-old plasmodia; lane 3, Western blot for 3-d-old plasmodia with antisera against DNA polymerase \( \delta \)-peptide; lane 4, labelling of the sample shown in lane 3; lane 5, Western blot with antisera against human PCNA for 2-d-old plasmodia (Percoll absent); lane 6, labelling of the sample shown in lane 5; lane 7, Western blot with antisera against human RP-A (70 kDa subunit) for 2-d-old plasmodia (Percoll absent); lane 8, labelling of the sample shown in lane 7. (c) Comparison of low-molecular-mass polypeptides for 2-d-old plasmodia after SDS-PAGE (15% acrylamide). Note that electroblotting of high-molecular-mass proteins was incomplete. Lane 1, Western blot with antisera against yeast RF-C subunit 4 (Percoll absent); lane 2, labelling of the sample as shown in lane 1; lane 3, labelling of the sample shown in lane 2, but in the presence of 25% (v/v) Percoll.

The presence of 25% (v/v) Percoll, a reagent routinely employed for the preparation of ‘clean’ nuclei by density-gradient centrifugation (e.g. Angerer & Holler, 1995), provoked leakage of proteins from the nuclei. DNA polymerase \( \epsilon \), 46 kDa PCNA-like protein, and 54 kDa RP-A-like protein (Fig. 7a, lanes 1–3) were identified. These proteins could be depleted from the nuclei (Fig. 7a, lane 3), except for DNA polymerase \( \epsilon \), which remained in an approximately equal amount in the washed nuclei (Fig. 7a, lanes 2 and 3). Percoll also enhanced the intensity of other labelled bands, some of them not found in its absence (e.g. Fig. 6c, lanes 2 and 3). Bands of DNA polymerase subunits indicated by arrows were one example (Fig. 6a, lane 7; Fig. 6b, lane 2; Fig. 7a, lane 3; Fig. 7b, lanes 1 and 3). Bands in positions 14.5–23 kDa (arrows in Fig. 6c, lane 3) with molecular masses reminiscent of \( P. \) polycephalum histones (Cote et al., 1985) provided another example. It has to be noted that in Fig. 6(c) bands at >100 kDa were not efficiently electroblotted due to the high concentration (15%) of polyacrylamide. The missing bands were, however, identified in the electrophoretic gel (not shown). The cytoplasmic fraction, assessed without Percoll addition, was devoid of labelled bands.

The observation that histones and certain subunits of DNA polymerases were not labelled in the extract when Percoll was absent raised a question about inaccessibility to photoreactive DNA. These proteins were presumed to be masked by participating in higher-order complex(es) of DNA polymerase holoenzymes, histones, and probably other polypeptides. Percoll disrupted the complex and restored access similar to that in purified preparations. A candidate agent that promoted the formation of such higher-order complexes was PMLA. We examined different concentrations of the polymer; the results for some of them are shown in Fig. 7(c). The effects on labelling were spectacular. At 5 \( \mu \)g endogenous PMLA ml\(^{-1}\) in the labelling mixture (arrows in Fig. 7c, lane 2), inhibition was relieved by the presence of 0.2 mM added spermine. HCl (Fig. 7c, lane 1) as described by Angerer & Holler (1995). At 75 \( \mu \)g ml\(^{-1}\) (Fig. 7c, lane 3) and higher PMLA concentrations, labelling of all but the two bands at 200 and 140 kDa was suppressed. The 200 kDa band was related to DNA polymerase \( \epsilon \) by immunostaining. The 140 kDa band was referred to \( \beta \)-like DNA polymerase because of its resistance to PMLA inhibition (Fischer et al., 1989).
concluded that the variation in labelling was due to the complex-forming activity of PMLA.

DNA-replicating polymerases are believed to be cleared from the nuclei concomitantly with growth arrest. Plasmodia start to arrest 2 d after inoculation, while the level of PMLA remains high until day 5 (Holler et al., 1992a). On that basis, the higher-order complexes of PMLA would have to be cleared from the nuclei. A comparison for 2 d plasmodia (Percoll present in Fig. 7a, lane 3; Percoll absent in Fig. 7a, lane 1, and Fig. 8, lanes 9 and 10) and 3 d plasmodia (Percoll present in Fig. 7b, lane 1; Percoll absent in Fig. 8, lanes 11 and 12) suggested that nicked DNA polymerases were accumulated in the nuclei. Immunostaining indicated that fragment bands were generated especially from DNA polymerases α and ε. Despite nicking, labelling activities were maintained in the nuclei similarly as during nicking of purified DNA polymerase ε in Fig. 5. Fragmentation/nicking was not observed during the mitotic cycle (data not shown). We assumed that it was essential for the removal of DNA polymerases from the nuclei. That nicking was not an experimental artefact is supported by the following arguments: (i) protease inhibitors were present during sample preparation; (ii) prolonged standing of nuclei or nuclear extract did not change the band patterns; (iii) if 3 d plasmodia contained proteolytic activities that introduced nicks during sample preparation, new bands would have been generated in extracts of 2 d plasmodia after mixing with 3 d plasmodia (Fig. 8, lanes 2–7). Also, mixing of the two nuclear extracts did not have this effect (Fig. 8, lanes 13 and 14).

**Conclusions**

The results for purified DNA polymerases indicated that their labelling and identification was possible. A labelled band of 230 kDa enabled us to isolate and identify *P. polycephalum* DNA polymerase ε. This type of polymerase has been shown to be present in yeast and metazoans (Hindges & Hübscher, 1997), and now in the myxomycete *P. polycephalum*. We have previously reported that the replicatively active DNA polymerases α and δ were inhibited by PMLA (Fischer et al., 1989; Achhammer et al., 1995). That DNA polymerase ε is also inhibited supports our assumption that PMLA forms...
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complexes with replicatively active proteins. This is further substantiated by comparing DNA polymerases in purified samples and in nuclear extracts of plasmodia. Photoaffinity labelling revealed that several of the subunits of the holoenzymes were in a more 'open' configuration when they were purified than in the nuclear extract. Complex formation with PMLA rendered them inaccessible for the cross-linking DNA. Percoll partially disrupted the complexes by a mechanism still to be clarified. The induction of leakage of particular proteins from the nuclei is in support of the complex formation. The existence of PMLA–DNA polymerase–histone complexes in plasmodial nuclei, even in the presence of Percoll, has been demonstrated (Angerer & Holler, 1995). Maintenance of the complexes could be the molecular basis for the reported PMLA homeostasis (Schmidt et al., 1996; Karl & Holler, 1998).

The fate of DNA polymerases when DNA was no longer replicated was studied at the arrest of plasmodial proliferation before differentiation to spherules (a cell form of the organism starving in the dark) or spores (starving in the light). DNA polymerases were nicked, but as such were still active in the nuclear extract before they finally disappeared. Nicking could provoke functions specific for growth termination of plasmodia, such as prevention of the re-entry of protein–PMLA complexes when exported from the nucleus.

We find that *P. polycephalum* DNA polymerase α, δ, and ε share similarities with the polymerases from higher eukaryotes. The results from immunostaining indicated conservation of epitopes in the case of the replication factors. The abundant 46 kDa PCNA-like polypeptide and the 54 kDa RP-A-like species are novel because of their unusual sizes. The replication apparatus may thus be similar to that of animals, fungi and plants. Observed special features such as binding to PMLA may account for the plasmodium-specific physiology.

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