Isolation and characterization of replication protein A (RP-A) from tobacco cells

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Abstract Replication protein A (RP-A) was isolated from tobacco suspension cells and purified to near homogeneity by a procedure involving isolation of protoplasts, preparation of nuclei, nuclear lysis, binding to a column of single-stranded (ss) DNA cellulose and elution at different salt concentrations. The purified protein contained three subunits with molecular masses of 70, 34 and 14 kDa, and was free from nuclease activity. Tobacco RP-A had a high affinity for ssDNA. Binding competition experiments indicated only a weak affinity for double-stranded DNA and no detectable affinity for ssRNA. Photochemical cross-linking experiments indicated that the 70 kDa subunit has the ssDNA-binding activity. Tobacco RP-A was able to stimulate the activity of a tobacco α-like DNA polymerase about 4-fold. This is the first isolation of RP-A from a plant and the procedure may be generally applicable to other plant species.

Key words: Tobacco; Replication protein A; DNA polymerase; Single-stranded DNA-binding protein

1. Introduction

Single-stranded (ss) DNA-binding proteins (SSB) have essential roles in DNA replication, recombination and repair in both prokaryotes and eukaryotes [1,2]. SSBs bind to and stabilise ssDNA intermediates generated during these processes, remove secondary structure from and configure the ssDNA templates for subsequent catalytic events, and interact with a variety of proteins involved in DNA metabolism, including helicases, nucleases and DNA polymerases [3–5]. *Escherichia coli* SSB is composed of four identical 18.9 kDa subunits [6], whereas the major nuclear SSB from vertebrate, invertebrate and yeast cells, termed replication protein A (RP-A), consists of three non-identical subunits of 70, 30–36 and 8–14 kDa [7–13]. In contrast to the intensively studied SSBs of bacterial, animal and yeast cells, little is known about plant SSBs and no plant RP-A has hitherto been reported. An SSB, termed helix-destabilizing protein or R-protein, isolated from meiotic cells of *Lilium*, was shown to stimulate a DNA polymerase activity in extracts of meiotic cells [14–16]. R-protein is distinguishable from RP-A, because it is composed of a single subunit of 35 kDa and because it is present in maximum amounts at the end of S-phase.

Here we report the isolation and characterization of an SSB from purified nuclei of tobacco suspension culture cells and show that it has the properties typical of an RP-A.

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Abbreviations: BSA, bovine serum albumin; DNase, deoxyribonuclease; ds, double-stranded; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol-bis(β-aminoethyl ether) *N,N,N′,N′*-tetraacetic acid; RP-A, replication protein A; ss, single-stranded; SSB, ssDNA-binding protein; HEPES, *N*-[2-hydroxyethyl]-piperazine-*N′*-[2-ethanesulphonic acid]; MES, 2-[morpholino]ethanesulphonic acid; PMSF, phenylmethylsulphonyl fluoride; SDS-PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; TCA, trichloroacetic acid; Tris, tris[hydroxymethyl]aminomethane

2. Materials and methods

2.1. Tobacco cell suspension culture

Tobacco BY-2 cells [17] were grown in Murashige Minimal Organics Medium (Life Technologies) supplemented with KH_2PO_4 (540 mg/l), thiamine (1 mg/l) and 2,4-dichlorophenoxyacetic acid (0.2 mg/l) in suspension culture shaken at 125 rev/min in the dark at 27°C. The culture was transferred to fresh medium (3:100) at weekly intervals. Cells were harvested at middle log phase (4 days after inoculation) by centrifugation at 200×*g* for 5 min.

2.2. Preparation of protoplasts from tobacco cells

Tobacco BY-2 cells (50 g) were resuspended in 400 mM mannitol (100 ml) and inverted to wash. The cell pellet was then resuspended in 150 ml of 0.5% MES–KOH, pH 5.6, containing 380 mM mannitol, 8 mM $CaCl_2$, 1% cellulase R-10 (Yakult Honsha Co. Ltd., Japan) and 0.1% macerozyme R-10 (Yakult Honsha Co. Ltd., Japan) and incubated at 22°C for 30 min at 50 rev/min, 16 h at rest, 30 min at 50 rev/min and 30 min at rest. Protoplasts were harvested by centrifugation at 200×*g* for 5 min and washed 3 times with ice-cold 400 mM mannitol, pH 5.6.

2.3. Preparation of tobacco nuclei

The tobacco protoplasts were suspended in 1 vol of ice-cold nuclear isolation buffer (30 mM HEPES–KOH, pH 7.9, 36% w/v Ficoll 400, 2% w/v polyvinylpyrrolidone, 20 mM NaF, 5 mM DTT, 2 mM EGTA, 1 mM EDTA, 0.3 mM spermine, 1 mM spermidine, 1 mM PMSF, 1 mM benzamide, 3 μg/ml pepstatin A and 2 μg/ml leupeptin) and 1:20 vol of 20% w/v freshly prepared skim-milk solution. Nuclei were then isolated as previously described [18], suspended in 20 mM HEPES–KOH, pH 7.8, 5 mM $MgCl_2$, 1 mM DTT, 250 mM sucrose, 50% v/v glycerol and stored at −80°C.

2.4. Isolation and purification of tobacco RP-A

Nuclei were thawed, harvested by centrifugation at 2500×*g* for 5 min at 2°C and suspended in 2 vol of 25 mM HEPES–KOH, pH 7.9, 25% v/v glycerol, 4 mM $MgSO_4$, 0.2 mM EGTA, 1.8 mM EDTA, 10 mM NaF, 5 mM DTT, 3 μg/ml pepstatin A, 2 μg/ml leupeptin and 1 mM PMSF. Nuclei were lysed by adding 4 M ammonium sulphate to a final concentration of 0.42 M and shaking gently at 0°C for 30 min. The lysate was centrifuged at 200 000×*g* for 1 h at 2°C and the supernatant was immediately dialysed twice for 3 h against 500 ml of buffer A (25 mM HEPES–KOH, pH 7.4, 10% v/v glycerol, 0.02% NP-40, 2 mM DTT, 1.0 μg/ml leupeptin, 1.0 μg/ml pepstatin A, 0.5 mM PMSF, 1 mM EDTA, 1 mM EGTA), containing 0.1 M NaCl, followed by centrifugation at 30 000×*g* for 30 min. The supernatant was loaded onto a 1×4 cm column of ssDNA cellulose (Sigma), equilibrated with buffer A containing 0.1 M NaCl. The column was washed sequentially with 5 vol of buffer A containing 0.75 M NaCl and 3 vol of buffer A containing 2.5 M NaCl. The peak fractions with DNA-binding activity (5 ml) were pooled, dialysed overnight against 2 l of 20 mM Tris-HCl, pH 7.4, 25% v/v glycerol, 0.01%

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NP-40, 1 mM DTT, 0.1 M NaCl, 1.0 μg/ml leupeptin, 1.0 μg/ml pepstatin A, 0.5 mM PMSF, concentrated using Centricon-3 concentrators and stored at −80°C.

2.5. SDS–polyacrylamide gel electrophoresis (SDS-PAGE)

This was carried out as described by Laemmli [19] with a 5% stacking gel and a 13% separating gel.

2.6. Labelling of nucleic acids

Phage M13mp18 was labelled by primer extension using the forward sequencing primer (Pharmacia), 0.1 mM each of dATPs, dGTP and dTTP, 20 μmol dCTP, 10 μCi [α-^{32}P]dCTP (3000 Ci mmol^{-1}) and 5 U of the Klenow fragment of *E. coli* DNA polymerase I. The labelled product, bound to its template, was denatured by heating to 100°C for 5 min followed by rapid cooling to 0°C.

2.7. Photochemical cross-linking

Purified tobacco RP-A (50 ng) was incubated with 1 ng of ^{32}P-labelled phage M13mp18 ssDNA or dsDNA in 30 mM HEPES–KOH (pH 7.5), 1 mM DTT, 100 mM KCl, 0.1 mg/ml BSA, 5 mM $MgCl_2$ in a total volume of 20 μl at 20°C for 1 h. The reactions were then subjected 3 times to 2 J of UV radiation in a Stratalinker 1800 (Stratagene). Mixtures were then adjusted to 5 mM $CaCl_2$ and 50 μg/ml DNase I, incubated at 30°C for 30 min and analysed by SDS-PAGE (12.5% gel). Bands were detected by autoradiography.

2.8. Effect of tobacco RP-A on tobacco α-like DNA polymerase activity

Reaction mixtures (30 μl) contained 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1.0 mM DTT, 5 μg BSA, 0.1 μg poly(dA).$(dT)_{12-16}$, 0.005 U of tobacco α-like DNA polymerase, 10 μM [^{3}H]TTP (5000 cpm/pmol), 8% v/v glycerol and various amounts of tobacco RP-A. RP-A and DNA polymerase were incubated for 5 min at 25°C. The other components of the reaction mixture were then added and the mixture was incubated at 35°C for 1 h. The reaction was stopped by addition of 1 ml of ice-cold 10% w/v TCA containing 50 mM sodium pyrophosphate. After incubation at 0°C for 20 min, the precipitates were collected on Whatman GF/C filters and washed with 15 ml of 5% TCA containing 50 mM sodium pyrophosphate, followed by 5 ml of ethanol. Radioactivity retained on the filters was determined by counting in a liquid scintillant (Ecoscint A, National Diagnostics). One unit of DNA polymerase activity corresponds to the incorporation of 1 nmol of TMP into acid-insoluble product in 1 h at 37°C.

2.9. Test for deoxyribonuclease activity in tobacco RP-A

Reaction mixtures (20 μl), containing 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 2.0 mM DTT, 5 μg BSA, 1 ng of ^{32}P-labelled phage M13mp18 dsDNA and tobacco RP-A (50 ng), were incubated at 37°C for various lengths of time and then analysed by electrophoresis through 0.7% agarose gels and autoradiography [20].

3. Results

3.1. Isolation and purification of tobacco RP-A

Attempts to prepare RP-A from homogenates of tobacco leaves or total tobacco cell extracts by methods used successfully for isolation of RP-A from *Drosophila* [21,22], mammalian [23,24] and yeast [8,25] cells were unsuccessful. This was due to the presence in total tobacco cell homogenates of large amounts of ssDNA-binding proteins, unrelated to RP-A, probably arising from chloroplasts and mitochondria. Successful isolation of tobacco RP-A was only achieved after first isolating nuclei from protoplasts prepared from tobacco suspension culture cells. Tobacco RP-A was separated from other nuclear DNA-binding proteins by chromatography on a column of ssDNA cellulose. Proteins which bound moderately strongly to ssDNA cellulose were eluted with 0.8 M NaCl. Tobacco RP-A which bound very tightly to ssDNA was subsequently eluted with 2.5 M NaCl. The yield of purified RP-A from 100 g wet weight of tobacco suspension cells was 1 μg.

Analysis of tobacco RP-A by SDS-PAGE (Fig. 1) indicated

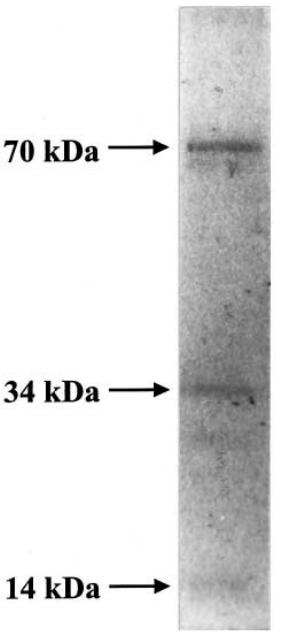

Fig. 1. SDS-PAGE analysis of tobacco RP-A. 0.1 μg of tobacco RP-A was separated in a 13% SDS–polyacrylamide gel and stained with silver. The positions and sizes of the three subunits (kDa) are shown on the side of the gel.

that it had been purified to near homogeneity. It consisted of three subunits of 70, 34 and 14 kDa. A small amount of a protein with a molecular mass of 28 kDa could be an impurity, a degradation product of the 34 kDa subunit, or a modified form or homologue of the 34 kDa subunit as found in HeLa cells [26].

3.2. Identification of the DNA-binding subunit of tobacco RP-A

Tobacco RP-A was incubated with ^{32}P-labelled M13mp18 ssDNA and the mixture was subjected to UV irradiation. After removal of uncross-linked DNA with DNase, the product was analysed by SDS-PAGE and autoradiography. Only one labelled band was detected which migrated with an apparent molecular mass of 72 kDa (Fig. 2, lane 3). No band of this size was detected in the absence of UV irradiation (Fig. 2, lane 2) or after treatment of the product with proteinase K. Proteins cross-linked to nucleotides generally migrate at rates corresponding to molecular masses 1–2 kDa greater than those of the free protein [27–29]. It is concluded that the 70 kDa subunit of tobacco RP-A has the ssDNA-binding activity.

3.3. Specificity of binding

The relative abilities of tobacco RP-A to bind to ssDNA, dsDNA and ssRNA were compared using binding competition experiments. Tobacco RP-A was incubated with ^{32}P-labelled M13mp18 ssDNA in the presence of 100-fold excesses of unlabelled M13mp18 ssDNA, M13mp18 dsDNA or cucumber mosaic virus ssRNA, the mixtures were subject to UV irradiation, and the products were analysed as described in Section 3.2. As can be seen in Fig. 3, the effect of 100-fold excess of unlabelled ssRNA is almost negligible (lane 2) when compared to the control lacking competitor (lane 1). In con-

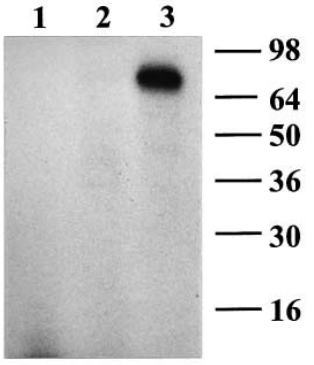

Fig. 2. Photochemical cross-linking of tobacco RP-A to ssDNA. Tobacco RP-A (50 ng) was incubated with 1 ng of ^{32}P-labelled phage M13mp8 ssDNA and then subjected to UV irradiation (lanes 1 and 3) or not irradiated (lane 2). After DNase digestion (lanes 1–3), followed by treatment (30 min at 37°C) with proteinase K (1 mg/ml, lane 1) or no proteinase K treatment (lanes 2 and 3), the products were analysed by SDS-PAGE (13% gel). Bands were detected by autoradiography. The sizes of marker proteins (kDa) are indicated on the right.

trast, 100-fold excess of unlabelled ssDNA reduced the cross-linking of tobacco RP-A to the labelled ssDNA to undetectable levels (lane 4). The effect of 100-fold excess of dsDNA was intermediate between that of ssRNA and dsDNA; cross-linking of tobacco RP-A to labelled ssDNA was reduced, but only about 3-fold (lane 3). It is concluded that the affinity of tobacco RP-A for ssDNA is much higher than that for dsDNA; the protein had only very weak, if any, affinity for ssRNA.

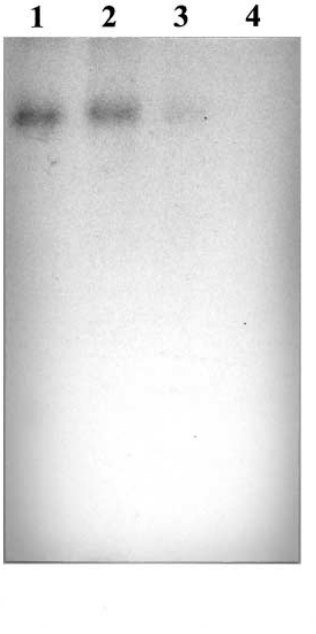

Fig. 3. Binding competition. Tobacco RP-A (50 ng) with no competitor (lane 1) or mixed with unlabelled cucumber mosaic virus ssRNA (100 ng, lane 2), M13mp18 dsDNA (100 ng, lane 3) or M13mp18dsDNA (100 ng, lane 4), was incubated with 1 ng of ^{32}P-labelled M13mp18 ssDNA and then subjected to UV irradiation. After DNase digestion, the products were analysed by SDS-PAGE (13% gel), followed by autoradiography.

3.4. Stimulation of the activity of a tobacco α-like DNA polmerase

We have recently isolated an aphidicolin-sensitive, α-like DNA polymerase from tobacco cells (unpublished results). SSBs often have the ability to stimulate DNA polymerase activity [1,3]. The effect of tobacco RP-A on the activity of the tobacco α-like DNA polymerase was therefore tested. Polymerase activity increased with increasing amounts of tobacco RP-A reaching a maximum of 4-fold stimulation (Fig. 4). Further increase in the amount of tobacco RP-A reduced the stimulation of polymerase activity to about 1.5-fold. Generally stimulation of DNA polymerase activity by mammalian and insect RP-As has continued to increase with increasing amount of RP-A [3,22]. The difference in this respect between the tobacco RP-A and RP-As of some other organisms may reflect different affinities of different RP-As for their cognate DNA polymerases and/or the ssDNA template. However to check that the reduction in stimulation of the tobacco DNA polymerase with amounts of tobacco RP-A greater than that required to give maximum stimulation was not due to the presence of trace amounts of nuclease in the RP-A preparation, the maximum amount of tobacco RP-A used in the DNA polymerase stimulation experiments was incubated with ^{32}P-labelled phage M13mp18 DNA for periods up to 16 h. Analysis of the DNA by agarose gel electrophoresis, followed by autoradiography, revealed no evidence of degradation of the labelled DNA (not shown).

4. Discussion

We have isolated a plant RP-A for the first time. The purification procedure, which requires prior isolation of the nuclei to remove ssDNA binding proteins from chloroplasts and mitochondria which would otherwise interfere with the procedure, should be applicable to isolation of RP-A from a wide range of plant species. This is an essential step in the con-

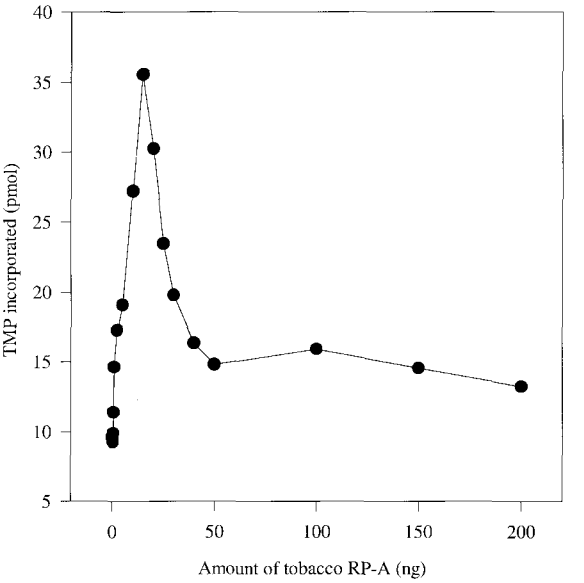

Fig. 4. Stimulation of DNA polymerase activity by tobacco RP-A. Tobacco α-like DNA polymerase, poly(dA).(dT)$_{12-16}$, [^{3}H]TTP and various amounts of tobacco RP-A were incubated at 35°C for 1 h. Incorporation of [^{3}H]TMP into TCA-insoluble product was then determined.

struction of in vitro plant DNA replication systems from defined components.

The tobacco RP-A shares several properties with RP-As isolated from mammalian, insect and yeast cells [3–5,7–13]. (i) It has three subunits of sizes similar to those of the RP-As of other organisms. (ii) It binds to ssDNA with high affinity, has lower affinity for dsDNA and has little or no affinity ssRNA. (iii) The largest of the three subunits has the DNA-binding activity. (iv) It has the ability to stimulate the activity of a cognate DNA polymerase. These similarities in properties to RP-As with proven function, together with its location in the nucleus, suggest that the tobacco RP-A plays roles in tobacco chromosomal DNA replication, recombination and repair similar to those of RP-As of mammals, insects and yeasts. Indeed it is likely that the functions of RP-As are conserved throughout the eukaryotes.

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