Disaggregation and Reconstitution of Oligomeric Complexes of Simian Virus 40 Large T-Antigen

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SUMMARY

Biochemical properties of multiple species of simian virus 40 (SV40) large T-antigen produced in SV40-infected monkey cells were investigated by zonal sedimentation centrifugation of radiolabelled cell extracts on sucrose gradients. Two major subpopulations of T-antigen detected by immunoprecipitation and gel electrophoresis could be distinctly separated: low molecular weight forms ranging approximately between 5S and 10S and higher oligomeric forms at about 16S to 23S. Removal of divalent cations by chelating agents such as EDTA disassembled higher oligomers into low molecular weight forms (5S to 10S). Adding divalent cations in excess of the EDTA concentration reassembled the higher oligomeric forms, showing a sedimentation behaviour like T-antigen in untreated cell extracts. Our results are compatible with the hypothesis that divalent cation binding properties of T-antigen participate in the natural pathway of assembling multiple oligomeric species.

Large T-antigen, encoded by the early region of the oncogenic simian virus 40 (SV40), is a multifunctional phosphoprotein (92000 $M_r$) which accumulates in the nucleus (Tooze, 1980) and is detected in small amounts on the surface of SV40-infected and -transformed cells (Soule et al., 1980; Deppert & Henning, 1980). It exists in multiple molecular species which can be characterized by variable phosphorylation (Edwards et al., 1979; Montenarh & Henning, 1980, 1982) or by various sedimentation coefficients ranging from 5-5S (monomer), 7S (dimer), 15.5S (tetramer) up to values higher than 28S as determined by analysing highly purified T-antigen (Griffin et al., 1979; Bradley et al., 1981, 1982) or estimated using whole cell extracts containing T-antigen (McCormick & Harlow, 1980; Harlow et al., 1980; Fanning et al., 1981a, b; Greenspan & Carroll, 1981; Prives et al., 1980). The monomeric T-antigen is a precursor for oligomeric species which are composed of either identical or non-identical subunits (Fanning et al., 1981b; Greenspan & Carroll, 1981). In the latter case, particularly T-antigen in higher oligomeric species (23S to 28S) is tightly bound to a host cell-encoded phosphoprotein (53000 $M_r$) which has been referred to as p53 (NVT, non-viral tumour antigen, Tau antigen, etc.) (McCormick & Harlow, 1980; Harlow et al., 1980; Fanning et al., 1981a, b; Greenspan & Carroll, 1981).

Although not yet conclusive, many observations suggest that the biological activities of SV40 T-antigen can be partially dictated by its molecular state, which in turn can be regulated by modulation of the monomer (phosphorylation) (Edwards et al., 1979; Montenarh & Henning, 1980, 1982; Fanning et al., 1981b; Greenspan & Carroll, 1981) and by environmental conditions influencing the assembly of oligomers. In an attempt to understand the protein–protein interactions leading to oligomeric T-antigen complexes, we studied the influence of divalent cations on the sedimentation behaviour of T-antigen extracted from lytically SV40-infected cells. Our observations indicate that native oligomeric subclasses of T-antigen are linked by divalent cations.

SV40-infected monkey cells were radiolabelled 48 h after infection for 4 h with [35S]methionine and extracted. Multiple forms of T-antigen were investigated by zonal sedimentation of whole cell extracts on 5 to 20% sucrose gradients. Each fraction of the gradient was immunoprecipitated with hamster SV40 tumour serum and analysed by SDS–polyacrylamide slab gel electrophoresis (SDS–PAGE). The sedimentation coefficients were estimated according to the sedimentation of correspondent marker molecules in parallel gradients such as ribosomal RNA.
or pure standard proteins. As shown in Fig. 1 (a) by an experiment which was representative of four experiments, various sizes of T-antigen seem to be distributed broadly over the gradient in different forms. Reproducibly, two prominent peaks were recognized: a major one at 5S to 10S and a smaller one showing a maximum around 16S. Minor amounts of T-antigen were detected in higher aggregated forms which are probably composed of T-antigen and p53 (53 000 Mr), as reported previously (Lane & Crawford, 1979; McCormick & Harlow, 1980; Harlow et al., 1980; Prives et al., 1980; Fanning et al., 1981 a, b; Greenspan & Carroll, 1981; Bradley et al., 1982). Depending on the labelling conditions, both p53 and small t-antigen were found but no other proteins were reproducibly detected in T-antigen-containing immunoprecipitates. These observations restrict the possibility that the sedimentation behaviour of T-antigen in whole cell extracts is generally influenced by co-migrating proteins. This statement agrees with data reported by Bradley et al. (1982) comparing sedimentation of T-antigen in whole extracts with that of highly purified T-antigen. Thus, for several reasons the use of whole cell extracts reduces loss of distinct subpopulations of T-antigen but does not distinctly influence the sedimentation behaviour.

In order to test the possible influence of divalent cations on the protein–protein interactions between subunits of oligomeric complexes of T-antigen, we analysed the sedimentation behaviour under identical conditions but in the presence of chelating agents (20 mM) such as EDTA or EGTA in cell extracts and sucrose density gradients. By comparison of Fig. 1 (b) with Fig. 1 (a), the majority of higher oligomeric forms of T-antigen seemed to disaggregate, yielding predominantly low molecular weight species of T-antigen. At lower concentrations of both chelating agents (5 mM-EDTA and -EGTA) we observed the beginning of the disassembly of higher oligomers (data not shown). At 20 mM, EGTA was as effective as EDTA. The sedimentation patterns of radiolabelled T-antigen shown in Fig. 1 (a, b) were confirmed by staining the gels with Coomassie Brilliant Blue (data not shown). Thus, the sedimentation behaviour of T-antigen radiolabelled for 4 h corresponded to that of the majority of T-antigen extracted from SV40-infected cells late in productive infection (48 h post-infection). To determine the influence of the ionic strength of the 20 mM-EDTA used for these experiments, we added NaCl instead of EDTA and found that up to a 0·3 M concentration NaCl had no influence on the sedimentation pattern shown in Fig. 1 (a) (data not shown).

According to these observations oligomeric T-antigen complexes can be disassembled by removal of divalent cations. The validity of this interpretation was proved by trying to

Fig. 1. Density gradient centrifugation of SV40 large T-antigen. TC-7 cells (4 × 10⁶) were infected with 1 ml undiluted SV40 virus stock (2×10⁶ p.f.u.). Forty-eight h post-infection cells were rinsed three times with methionine-deficient DMEM and labelled for 4 h with 30 μCi [35S]methionine. After labelling, the washed cells were lysed with 400 μl of extraction buffer [0·5% of the non-ionic detergent Nonidet P40 (NP40; Fluka), 10 mM-Tris–HCl pH 9, 0·1 mM-NaCl] for 30 min on ice. 10% Trasylol (Bayer) and phenylmethylsulphonyl fluoride (PMSF), L-(1-tosylamide-2-phenyl)ethyl chloromethyl ketone (TPCK) and Nα-p-tosyl-l-lysine chloromethyl ketone (TLCK) were added to a final concentration of 0·25 mg/ml. The lysate was centrifuged for 30 min at 105 000 g in a Beckman rotor type 50 at 4 °C. A 0·4 ml amount of the supernatant corresponding to 8 × 10⁶ cells was loaded on top of 10 ml linear 5 to 20% sucrose gradients (10 mM-Tris–HCl pH 7·3, 0·1 mM-NaCl, 0·5% NP40) layered on a cushion of 0·4 ml 20% sucrose and centrifuged for 12 h at 36 000 rev/min in an SW41 Beckman rotor at 4 °C. 23S, 16S and 5S ribosomal RNA (Boehringer, Mannheim) or, alternatively, catalase (10S), goat IgG (7S) and bovine serum albumin (4.3S) (Boehringer, Mannheim) were run in parallel gradients as markers. T-antigen was immunoprecipitated from 0·5 ml gradient fractions using 10 μl hamster SV40 tumour serum or normal hamster serum and 200 μl of a 10% suspension of heat-inactivated and formaldehyde-fixed Staphylococcus aureus (Cowan I) as described by Kessler (1975). After washing five times with NET buffer (1% NP40, 50 mM-Tris–HCl pH 7·4, 0·15 mM-NaCl, 5 mM-EDTA) and finally once with 50 mM-NH4HCO3, the immunocomplexes were eluted with 200 μl elution buffer (50 mM-NH4HCO3, 2% SDS, 2% 2-mercaptoethanol; 30 min incubation at 4 °C), lyophilized and dissolved in 20 μl sample buffer (65 mM-Tris–HCl pH 6·8, 5% 2-mercaptoethanol, 1% glycerol, 0·1% bromophenol blue; 10 min incubation at 100 °C). SDS–PAGE was performed on 1 mm slab gels as described by Laemmli (1970) and prepared for fluorography as described by Bonner & Laskey (1974). 14C-labelled methylated standard proteins (Amersham) were: ovalbumin (43 000 Mr), bovine serum albumin (68 000 Mr) and phosphorylase a (94 000 Mr). (a) Normal cell extract; (b) cell extract pretreated with 20 mM-EDTA; (c) cell extract in the presence of 20 mM-EDTA followed by 22 mM-CaCl2.
Fig. 2. Immunoprecipitation of SV40 large T-antigen in the presence and absence of EDTA. Extracts of $4 \times 10^6$ [$^{35}$S]methionine-labelled SV40-infected TC-7 cells (48 h post-infection) were immunoprecipitated either with 10 µl normal hamster serum (N) or 10 µl hamster SV40 tumour serum (T) in the presence (+) or absence (−) of 20 mM-EDTA. The immunoprecipitates were analysed by electrophoresis on a 10% SDS-polyacrylamide gel. Molecular weight markers are the same as described in Fig. 1. T-Ag, SV40 large T-antigen.

Reassemble the higher oligomeric forms of T-antigen by divalent cations. Therefore, we analysed the zonal sedimentation of T-antigen pretreated in cell extracts by 20 mM-EDTA and after addition of a slight excess of CaCl$_2$ or MgCl$_2$ up to a final concentration of 22 mM. The sedimentation pattern shown in Fig. 1(c) indicated that low molecular weight T-antigen can reaggregate into oligomeric complexes resulting in a pattern similar to the original pattern shown in Fig. 1(a). These results were again confirmed in control experiments by staining the T-antigen bands with Coomassie Brilliant Blue. It should be noted that the gradients in Fig. 1(a to c) were obtained by using the same amounts of aliquots of labelled extracts and all three gels were exposed for 24 h. The bands running at a mol. wt. of approximately 48 000 mainly detected in lanes 1 and 2 of Fig. 1(a, c) reflect non-reproducible impurities in the immunoprecipitates and not the p53 protein.

To exclude the possibility that 16S and higher oligomeric T-antigen might not be detected on sucrose density gradients by antisera after removal of divalent cations and, therefore, the gradients shown in Fig. 1 cannot be compared at a quantitative level we compared the yield of T-antigen obtained by immunoprecipitation from crude cell extracts in the presence or absence of 20 mM-EDTA. As shown in Fig. 2 the intensity of T-antigen-containing bands clearly demonstrates that pretreatment of cell extracts with 20 mM-EDTA does not decrease the total yield of T-antigen detectable with antibodies confirming observations of Smith et al. (1978).
Identical results were obtained in the presence of 20 mM-EDTA/22 mM-Ca$^{2+}$ (data not shown).

Our results suggest a new biochemical property, in that low molecular weight complexes reveal divalent cation binding properties leading finally to a self-assembly of higher oligomeric complexes of this protein. Modulations of T-antigen such as phosphorylation could increase its divalent cation-binding properties leading finally to the assembly of oligomers. On the other hand, various functional properties of T-antigen could be expressed or regulated depending on the concentrations of T-antigen, divalent cations or other environmental conditions. Thus, the host cell could influence equilibria dictating the processes of molecular assembly and disassembly. The question as to whether the divalent cation induced \textit{in vitro} reassembly of disaggregated T-antigen species induced by divalent cations follows the native pathway cannot yet be answered. The general biological importance of the assembly of oligomers has been demonstrated by analysing crude extracts of monkey cells infected with the temperature-sensitive SV40 mutant \textit{tsA} 58. At non-permissive temperature these cells produced only the 5S to 6S species of T-antigen which was unable to initiate SV40 DNA synthesis (Fanning \textit{et al.}, 1981\textit{b}; Greenspan & Carroll, 1981). Hence, the \textit{in vivo} self-assembly of oligomeric species seems to lead to numerous functional properties. The biological properties of T-antigen after disaggregation as well as after reassembly \textit{in vitro} shown in the present study remain to be elucidated.

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\textbf{REFERENCES}


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