Chemical crosslinking of different forms of the simian virus 40 large T antigen using bifunctional reagents

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Chemical crosslinking was used for a direct analysis of the different forms of large tumor (T) antigen, the simian virus 40 A gene product. The first subclass, sedimenting at 14–16 S, is composed of monomeric to tetrameric units, whereas the second, sedimenting at 5–6 S, only contains dimers and monomers of T. The occurrence of oligomeric structures of T in solution which are higher than dimers suggests the possibility of direct binding of such trimers or tetramers to the origin of replication of the viral DNA as an alternative to the formation of these structures by aggregation of bound dimers or monomers after their sliding along the DNA.

SV40 *Large T antigen* *Crosslinking* *Oligomer* *DNA*

1. INTRODUCTION

The SV40 A gene product, large T antigen, is required for both viral replication in productive infection of permissive hosts and oncogenic transformation of non-permissive cells. The large T antigen is a multifunctional protein whose binding to SV40 DNA was extensively analysed by several authors [1–5]. A great deal of effort has been expended to define the precise nature of specific recognition and binding of this protein to several sites of the viral DNA. Binding of large T antigen to at least one of these sites is an essential step in the initiation of viral DNA replication [6,7].

It has been recently shown that different subclasses of large T antigen from productively infected cells have different binding affinities for specific and non-specific DNA sequences of viral and cellular origin. The speculation was made that the isolated 14–16 S subclass could correspond to a tetrameric form of the large T antigen, whereas that of 5–6 S could be a dimeric one [8].

Investigations concerning the D2-T antigen, a polypeptide structurally and functionally related to the large T antigen, allowed one to conclude that this protein may exist most commonly in a tetrameric form [9]. These electron microscopic studies also favoured the existence of monomeric and dodecameric units.

However, efforts to determine the multimeric state of the authentic large T antigen in solution have led as yet to no real consensus. The nature of the different forms of this protein before binding was not demonstrated. This is nevertheless a prerequisite for a better understanding of the binding of these forms to the viral DNA.

Here, a study of these forms using chemical crosslinking techniques is reported.

2. MATERIALS AND METHODS

Basically, the method of Fanning et al. [8] was

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Abbreviations: DMS, dimethylsuberimidate; DTSP, dithiobis(succinimidyl propionate); SV40, simian virus 40; DTT, dithiothreitol; NEM, *N*-ethylmaleimide; PMSF, phenylmethylsulfonyl fluoride

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followed for isolation of the different forms of large T antigen. A fresh whole cell extract of SV40-infected CV-1P monkey kidney cells labeled for 2 h with ^{32}P (500 μCi/plate ∅ 150 mm) was layered on a 5–20% sucrose density gradient in 10 mM Hepes (pH 7.8), 150 mM KCl, 1 mM DTT, 0.5 mM $MgCl_2$, 1 mM PMSF and centrifuged at 45000 rpm at 0°C in an SW 55 rotor for 3 h. Each fraction of the gradient was immunoprecipitated, analyzed on an SDS-polyacrylamide gel and autoradiographed.

The fractions of a second gradient run in parallel and corresponding to the 14–16 S and 5–6 S forms were pooled and brought to 800 μl with 50 mM triethanolamine (pH 7.8), 1 mM $MgCl_2$ and used in crosslinking experiments.

Crosslinking was assayed with a 200 μl aliquot of the preparation of isolated forms, mixed with 20 μl DMS at different concentrations in 50 mM triethanolamine (pH 7.8), 1 mM $MgCl_2$ or with 200 μl DTSP diluted in 50 mM triethanolamine (pH 8.5), 1 mM $MgCl_2$. The final concentrations in the assays were 0.5, 5, 10, 50 mM DMS or 0.005, 0.01, 0.075, 0.1, 1 mM DTSP. After 15 min incubation at room temperature, 20 μl of a suspension of *Staphylococcus aureus* cells was added, and the immunoprecipitation was continued as described [10].

For the DTSP assay, the pooled fractions were treated for 20 min at 0°C with NEM (5 mM final concentration) and crosslinked for 15 min at 23°C; the reaction was stopped by adding 40 μl of 1 M lysine (100 × excess) and followed by immunoprecipitation, as above. For the isolation and identification of the crosslinked products, each probe was separated on a 3.5% SDS-

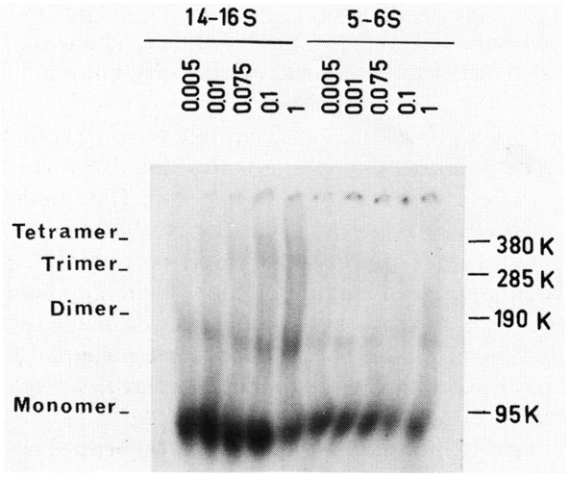

Fig.1. Crosslinking by DTSP of the purified subclasses (14–16 S and 5–6 S) of large T antigen isolated from SV40-infected CV-1P monkey kidney cells. A whole-cell extract of SV40-infected CV-1P cells labeled with ^{32}P was layered on a 5–20% sucrose gradient in 10 mM ethanolamine (pH 7.8), 150 mM KCl, 0.5 mM $MgCl_2$ and centrifuged at 0°C for 3 h and at 45000 rpm. The fractions were collected and pooled. The fractions corresponding to the 14–16 S and 5–6 S subclasses were adjusted to 800 μl with 50 mM triethanolamine (pH 8.5), 1 mM $MgCl_2$ and treated for 20 min at 0°C with NEM (5 mM final concentration). 200 μl aliquots of each form were incubated with an equal volume of different dilutions of DTSP in the same buffer for 20 min at 23°C (final concentrations of DTSP: 1, 0.1, 0.075, 0.01, 0.005 mM). The reaction was stopped by adding a 100-fold excess of lysine (1 M). The samples were immunoprecipitated, analysed on a 3.5% SDS-polyacrylamide gel and the dried gel autoradiographed. Crosslinked phosphorylase *a* was used as a marker for the theoretical values of T oligomers. K, kDa.

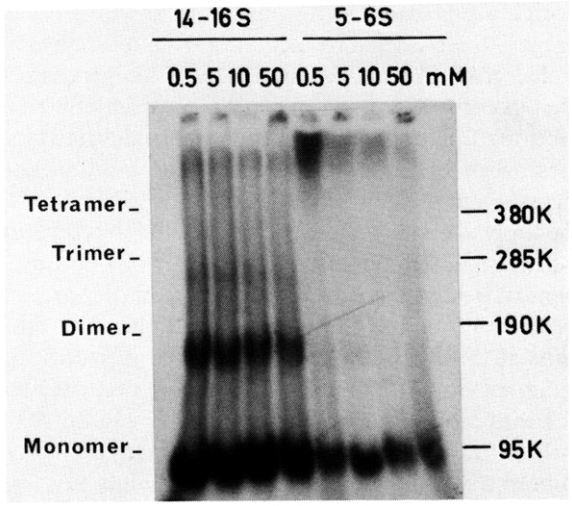

Fig.2. Crosslinking by DMS of the purified subclasses (14–16 S and 5–6 S) of large T antigen isolated from SV40-infected CV-1P monkey kidney cells. 200 μl aliquots of each subclass, adjusted to 800 μl with 50 mM triethanolamine (pH 7.8), 1 mM $MgCl_2$, were incubated with 20 μl of dilutions of DMS in the same buffer for 15 min at room temperature (final concentration of DMS: 50, 10, 5, 0.5 mM). 20 μl of a suspension of five times washed *S. aureus* cells was added, and the samples were processed as described in fig.1.

polyacrylamide gel [11] which was dried and autoradiographed at −80°C. 30 μl of crosslinked phosphorylase *a* [12] was used as a marker.

3. RESULTS AND DISCUSSION

Fig.1 shows the extent of crosslinking of the different forms by DTSP (12 Å span) as a function of the crosslinker added. The 14–16 S subclass contains monomeric to tetrameric forms of the large T antigen. The tetrameric form appears with a greater intensity at the higher concentrations used (0.1 and 1 mM DTSP) whereas the forms present in the 5–6 S subclass are predominantly monomeric, and, to a smaller extent, dimeric.

Fig.2 shows the results obtained with DMS (11 Å span). The use of this second type of crosslinker allows one to observe monomeric to trimeric forms of large T antigen in the 14–16 S subclass. The tetrameric form is not clearly visible. Interestingly, crosslinking with DMS seems to generate oligomeric forms of higher order than tetramers. In each case, the different bands of crosslinked large T antigen have molecular masses which are a little lower than those shown for the large T oligomers and calculated from a theoretical value of 95 kDa for the monomer. This is probably due to the fact that large T antigen, as often observed, is partially proteolysed, giving rise to a heterogeneous population which is nevertheless crosslinked. Minor binding forms of T (hexamer, octamer and dodecamer) were recently detected by Mastrangelo et al. [13] which might correspond to the population of higher order than tetramers visualized here. Using scanning transmission electron microscopy, they also showed a simultaneous binding of seven monomer-equivalent masses: three in region I and four in region II at the origin of replication of SV40 DNA. DMS does not allow one to obtain dimeric forms in the 5–6 S subclass with the same yield as DTSP.

From the data obtained by direct chemical crosslinking experiments of the unbound large T antigen in solution, it can be concluded that the 14–16 S subclass is composed of monomers, dimers, trimers and tetramers. This result is in accordance with the results of Mastrangelo et al. who demonstrated that such structures, preexisting in solution, are able to bind to DNA. In particular, a tetramer binds region II whereas a trimer is the largest structure observed in region I. The 5–6 S subclass is composed of monomers, and probably of a few dimers of large T antigen.

The 5–6 S forms serve as a precursor for the faster sedimenting forms. This conversion is defective at non-permissive temperature in tsA-infected cells [10]. Thus, the newly synthesized 5–6 S form would aggregate, generating the 14–16 S oligomeric form, which would bind more firmly to the viral DNA. This process appears to be essential for the initiation of replication [8].

A working model was recently proposed [8] to explain the binding of the large T antigen forms to the origin of replication of SV40 DNA. The newly synthesized 5–6 S, possibly a dimer, could transiently bind to the intracellular DNA and scan it until the origin sequence is recognized and specifically bound. T antigen may then aggregate, possibly on the DNA, into a 14–16 S form which is more firmly bound to the DNA. This model, based on the hypothesis that the 14–16 S subclass is a homogeneous population of tetramers, whereas the 5–6 S subclass might be composed of dimers, probably has to be revised.

The recent discovery of Mastrangelo et al., namely that the largest structure in region I is a trimer, is consistent with the possibility of the existence of trimers in solution shown here. These results make obsolete the affirmation that a dimeric or tetrameric structure of large T antigen interacts at each site [5].

It is not yet possible to deduce whether such a trimer binds directly to region I or results from the aggregation of a dimer and a monomer on the DNA. Despite further work needed for the understanding of the dynamics of binding of large T antigen on the SV40 genome, crosslinking appears to be a straightforward method to study the nature of large T antigen in solution.

Oligomers formed by large T antigen variants produced by transformed cell lines that are unable to initiate viral replication [4] could also be studied using the conditions described above. The fact that oligomerization of SV40 large T antigen may be involved in viral DNA replication is also of interest [14].

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