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The phosphorylation at Thr 124 of simian virus 40 large T antigen is crucial for its oligomerization

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SV40 large T antigen is phosphorylated at up to ten different amino acids clustered in an N-terminal and a C-terminal part of the polypeptide chain. The N-terminal phosphorylated residues include Ser 123 and Thr 124. We have analyzed the oligomerization, the complex formation with the cellular oncoprotein p53 and the DNA-binding properties of T antigen from two different SV40 transformed cell lines which have either an amino acid exchange at Ser 123 to Phe (W7) or Thr 124 to Ile (D29). In comparison to wild-type T antigen both mutant T antigens have a slightly reduced binding affinity for both binding sites, I and II, of SV40 DNA. Phosphorylation at both residues of T antigen is not essential for formation of the complex with p53. Only the phosphorylation at Thr 124 seems to be critical for the formation of high molecular mass oligomers. Our data support the hypothesis that the oligomerization of T antigen seems to be implicated in viral DNA replication.

SV40 large T antigen; Phosphorylation; Oligomerization; T-p53 complex formation; DNA binding

1. INTRODUCTION

The large T antigen of the DNA tumour virus SV40 is required for replication of the virus in permissive cells as well as for transformation of nonpermissive cells in culture (review [1]). The protein exists in monomers and various homologous oligomers [2–4] and also in heterologous complexes with the cellular oncoprotein p53 (review [5]). According to previous results the homologous oligomerization of T antigen seems to be involved in viral DNA replication but not in the maintenance of cell transformation [6–8]. On the other hand, T-p53 complexes seem to play a role in SV40 cell transformation [6].

T antigen is known to bind to three different sites of SV40 DNA. Site II spans the origin of SV40 DNA replication, site I is located on the early side of the origin and site III is on the late side.

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Binding of T antigen to site I is believed to block transcription of SV40 early mRNA, resulting in autoregulation of T antigen synthesis. Binding to site II is essential for the initiation of replication of virus DNA, while the consequences of site III binding are not yet fully understood [9–11].

The 708 amino acid long T antigen molecule is phosphorylated at up to ten serine and threonine residues which reside at both ends of the polypeptide chain. The amino-terminal cluster consists of Ser 106, 111, 112, 123 and Thr 124, while the carboxy-terminal region includes Ser 639, 676, 677, 679 and Thr 701 [12]. Although the phosphoamino acids have been identified, their functional significance remains to be elucidated. Several laboratories have shown that differential phosphorylation of T antigen seems to be involved in the regulation of its DNA-binding activities [13,14], oligomerization [13], T-p53 complex formation [4,15] and DNA replication [16]. By analyzing a variety of different deletion mutants of SV40 large T antigen we were able to exclude a functional influence of all C-terminal and of the

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N-terminal phosphorylation sites Ser 106, Ser 111 and Ser 112 on oligomerization and T-p53 complex formation [17]. From these analyses it remains possible that the phosphorylation at Ser 123 or Thr 124 might be important for oligomerization and/or T-p53 complex formation. Although the DNA-binding domain of T antigen has been mapped from amino acid 132 to 225 [18], phosphorylation of both amino acids might indirectly influence the binding to individual binding sites on SV40 DNA. Therefore, we have analyzed T antigen from two point mutants, W7 and D29, where phosphorylation at Ser 123 (W7) or Thr 124 (D29) is affected, for these properties.

2. MATERIALS AND METHODS

2.1. *Cells*

W7 and D29, SV40 transformed Rat-1 cells, were a kind gift from E. Paucha (Dana-Farber Cancer Institute, Boston, USA). T antigen from W7 cells as an exchange of His 122 to Tyr and Ser 123 to Phe, and D29 of Thr 124 to Ile [18,19]. All cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal calf serum.

2.2. *Radiolabelling and extraction of cells*

$3–4 \times 10^6$ cells were radiolabelled for 2 h with 148×10^4 Bq (40 μCi) [^{35}S]methionine in methionine-free DMEM. After labelling, the cells were washed with phosphate-buffered saline, scraped off the plates and lysed with 0.4 ml extraction buffer (0.5% Nonidet P-40, 10 mM Tris-HCl, pH 9.0, 0.1 M NaCl) [7]. The cell extract was clarified by centrifugation at $105000 \times g$ in a Beckman rotor (type 50) at 4°C.

2.3. *Sucrose density gradient centrifugation and immunoprecipitation*

A total of 0.4 ml cell extract was sedimented through a linear 5–20% sucrose density gradient. Each fraction of the gradient was analyzed for T-p53 complexes by the addition of monoclonal antibody PAb 122 directed against p53 [20] or by immunoprecipitation with PAb 108, a monoclonal antibody against T antigen [21], followed by incubation with heat-inactivated and formaldehyde-fixed *Staphylococcus aureus* as described [22]. Immunoprecipitates were washed, eluted, electrophoresed on 10% SDS-polyacrylamide gels and fluorographed as in [7].

2.4. *DNA-binding assay*

Equal amounts of immunoprecipitated T antigen were washed three times with binding buffer [80 mM KCl, 10 mM Hepes, pH 7.8, 1 mM dithiothreitol (DTT), 1 mg/ml bovine serum albumin and 0.1 mM EDTA] and resuspended in 250 μl binding buffer together with 2 μg sonicated calf thymus DNA and 0.4 μg ^{32}P-end-labelled restriction fragments containing either only site I (pKB1) or site II (pdl 1085). After incubating the reaction mixture for 30 min at 37°C, bound fragments were separated from free DNA by washing the binding complexes three times with NET buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.05% Nonidet P-40). To determine the percent specific binding, Cerenkov radiation of the input DNA and of that bound to the immunocomplex after washing was counted.

3. RESULTS AND DISCUSSION

It is well accepted that binding of T antigen to the ori region of SV40 DNA is required for viral DNA replication [23,24]. Since T antigen from D29 cells with the point mutation at Thr 124 is unable and since W7 T antigen with its mutations at His 122 and Ser 123 has a diminished capacity for supporting viral DNA replication, we analyzed the affinity of both T antigens separately for

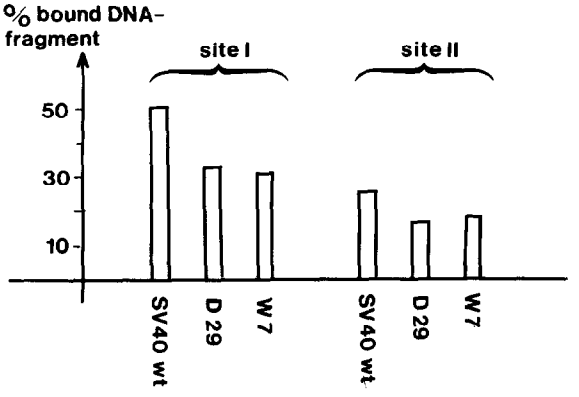

Fig.1. Specific DNA binding of T antigen from D29, W7 and SV40 wild-type infected cells. Immunopurified T antigen from D29, W7 or SV40 wild-type infected cells was assayed quantitatively for specific binding to binding site I cloned in pKB1 or site II cloned in pdl 1085. The ratio of bound to input DNA fragment was determined by Cerenkov counting.

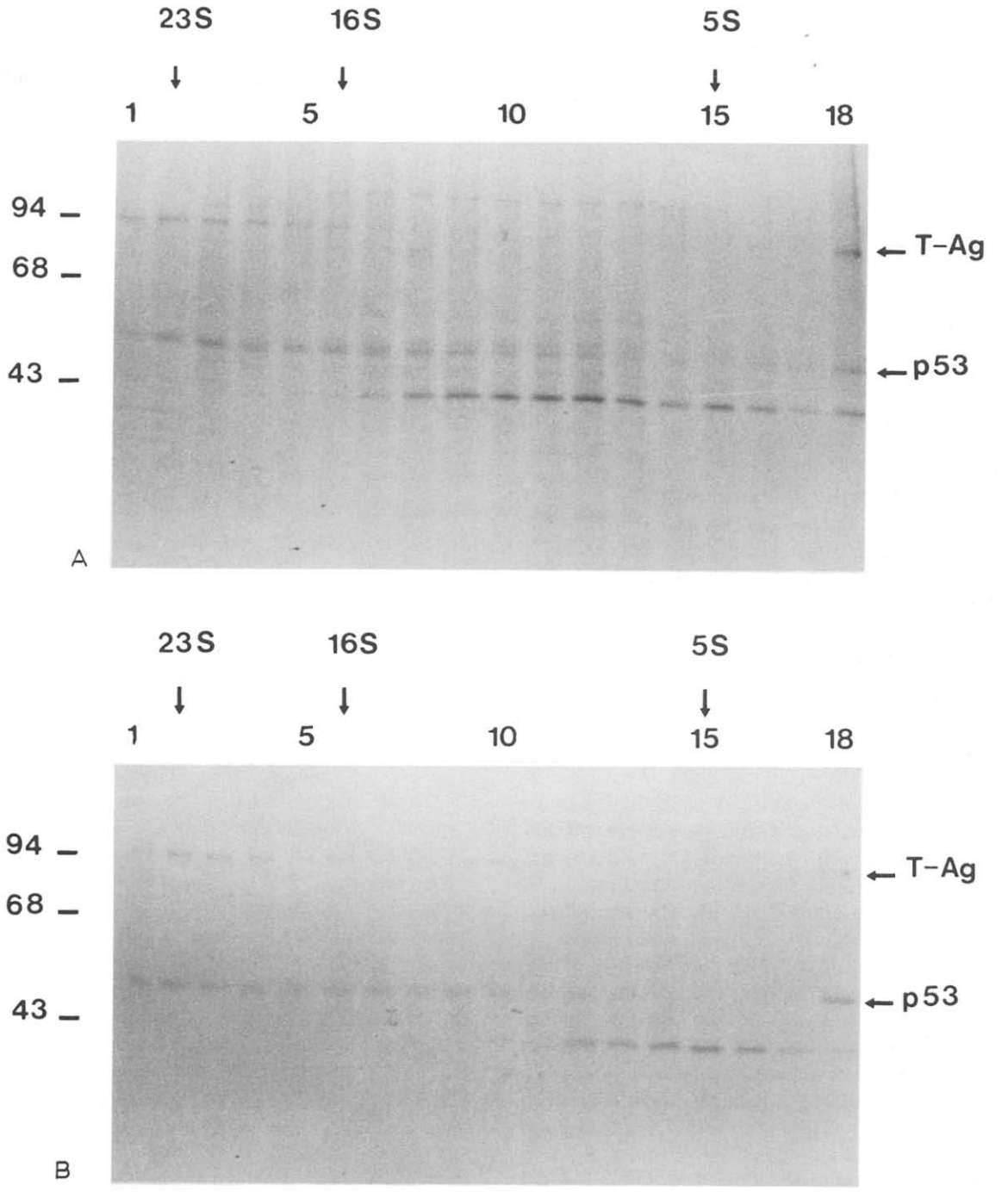

Fig.2. Sedimentation profiles of T-p53 complexes from W7 (A) or D29 (B) cells. Cells were labelled with [^{35}S]methionine and extracts were analysed by 5–20% sucrose density gradient centrifugation. Fractions of each gradient were immunoprecipitated with PAb 122 directed against p53. Immunoprecipitates were analysed on 10% SDS-polyacrylamide gels and fluorographed as in [7]. Markers for gradient centrifugation were rRNAs (5, 16 and 23 S). Markers for SDS-polyacrylamide gel electrophoresis were phosphorylase *a* (94 kDa), bovine serum albumin (68 kDa) and ovalbumin (43 kDa).

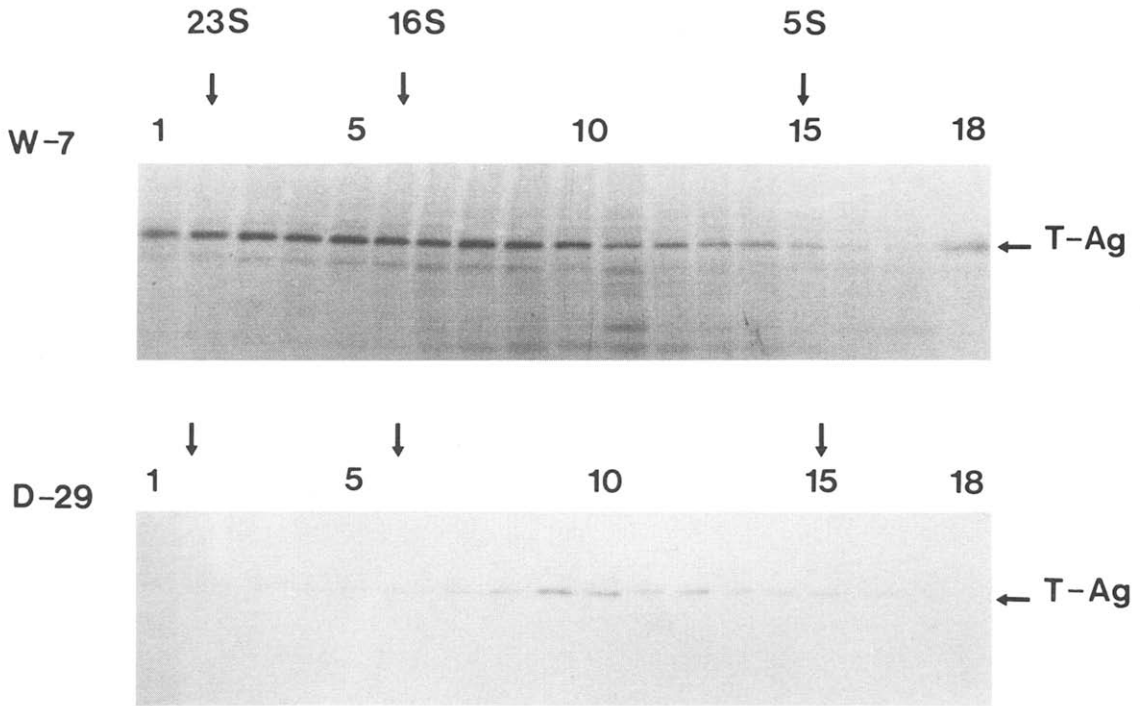

Fig.3. Sedimentation profile of free T antigen from W7 or D29 cells. Cell extracts from [^{35}S]methionine-labelled W7 or D29 cells were analyzed on 5–20% sucrose density gradients. After immunoprecipitation of each fraction with PAb 122 the supernatants of this first immunoprecipitation were further incubated with hamster SV40 tumour serum. Immunoprecipitates were analyzed on 10% SDS-polyacrylamide gels and fluorographed as in [7]. Markers are described in fig.2.

binding to site I or site II of SV40 DNA. For comparison T antigen from wild-type SV40-infected TC7 cells was bound to the same DNA fragments. We have used two different recombinant plasmids: pKB1 represents pBR322 with a cloned synthetic oligonucleotide of the 23 bp contact region of binding site I of SV40 DNA (nucleotides 5186–5208 of SV40 DNA) (Müller et al., submitted). pdl 1085, a kind gift from K. Peden, contains binding site II and short additional SV40 specific sequences in early and late directions (nucleotides 5210–133 of SV40 DNA) [25]. In order to quantitate even small differences in the binding of T antigen, we used a modified binding assay (Müller et al., submitted). Immunopurified T antigen from W7 cells, D29 cells or wild-type infected monkey cells was incubated under highly stringent conditions (80 mM KCl, pH 7.8) with only the ^{32}P-radiolabelled restriction fragment containing site II or site I and the DNA was quantitated before and after binding by Cerenkov radiation. Fig.1 shows the result of a typical experiment with the specific fragments of pdl 1085 or pKB1. T antigen from W7 and D29 cells showed a 1.5-fold reduced affinity for site I and also for site II when compared to wild-type T antigen. These results demonstrate that, although W7 T antigen is delayed and D29 T antigen is unable to support viral DNA replication [19], there is only a slight reduction in the binding to site II and also to site I. The reduced binding affinity of W7 T antigen as compared to that of the wild type might explain the delay in supporting viral DNA replication but does not explain why D29 T antigen is completely incapable of performing this function.

In order to evaluate the importance of phosphorylation at the amino acid Ser 123 or Thr 124 for the formation of high molecular mass complexes of T antigen with the cellular oncoprotein p53 and for the oligomerization of free T antigen, we have analyzed T-p53 complexes and free T antigen from W7 and D29 cells by sucrose density

centrifugation. Fig.2A and B shows the sedimentation profiles of free p53 and p53 complexed to T antigen from W7 and D29 cells, respectively. In both cases, T-p53 complexes were found sedimenting from 12 S towards the bottom of the gradients. These sedimentation profiles resemble those described for T-p53 complexes from several other SV40-transformed cell lines [2,3,6,15]. Thus, phosphorylation of Ser 123 and Thr 124 does not seem to be important for the formation of high molecular mass T-p53 complexes.

However, there is a considerable difference in the sedimentation profiles of free T antigen. As shown in fig.3, free T antigen from W7 cells formed predominantly high molecular mass oligomers, whereas free T antigen from D29 cells was found sedimenting mainly between 5 S and 14 S, i.e. free T antigen from D29 cells fails to form high molecular mass oligomers.

In summary, phosphorylation at Ser 123 influences neither the formation of high molecular mass T-53 complexes nor the oligomerization of free T antigen. However, phosphorylation at Thr 124 seems to be crucial for the formation of high molecular mass aggregates of free T antigen. Phosphorylation at both amino acids seems to influence the binding affinity of T antigen for binding site II and for I. In comparison to wild-type T antigen there is a reduction in binding affinities for both sites. This reduction, however, cannot explain the complete inability of D29 T antigen to support viral DNA replication.

Recent reports have shown that the oligomerization of T antigen is somehow involved in viral DNA replication [7,8]. Detailed structural analysis of T antigen from a temperature-sensitive A gene mutant of SV40 revealed that T antigen is simultaneously sensitive for DNA replication and for the formation of homologous oligomers but not for binding to SV40 or DNA [7]. Furthermore, analysis of the oligomerization of T antigen before and after the onset of viral DNA replication has shown a deceleration of the oligomerization late in infection, i.e. at the maximum of viral DNA replication [8]. From these data and from the results presented here, it seems likely that the defect of D29 T antigen in the formation of high molecular mass oligomers is responsible for the inability to function in viral DNA replication.

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