Regions of SV40 large T antigen necessary for oligomerization and complex formation with the cellular oncoprotein p53

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The simian virus 40 (SV40) T antigen is composed of 708 amino acids and forms monomers and various oligomers and, in small amounts, heterologous complexes with the cellular oncoprotein p53 (T-p53). Using SV40 mutants coding for T antigen fragments which are either deleted in the N-terminal half or truncated by various lengths at the C-terminal end, we found that a region between amino acids 114 and 152 and a C-terminal region up to amino acid 669 are essential for the formation of high M_r oligomers of T antigen. Furthermore, only the C-terminal end up to amino acid 669 is essential for T-p53 complex formation but not the N-terminus up to amino acid 152.

SV40 mutant *SV40 large T antigen* *Oligomerization* *T antigen-p53 complex* *Functional region*

1. INTRODUCTION

The simian virus 40 large tumour antigen (SV40 T antigen) is a multistructural protein which is involved in various steps in viral SV40 replication as well as SV40 cell transformation (reviews [1,2]). This protein is phosphorylated in multiple N- and C-terminal locations [3] and occurs structurally in different forms such as monomers (5 S) and various homologous oligomers (dimers, 7 S; tetramers, 16 S; higher oligomers, >16 S) [4]. Furthermore, it forms heterologous complexes with the cellular protein p53, called T-p53 (review [5]). Using different experimental approaches it has been shown that the oligomerization of T antigen seems to be somehow involved in viral DNA replication [6,7] but not in the maintenance of cell transformation [8]. On the other hand, the T-p53 complexes may be necessary but are certainly not alone sufficient for the maintenance of cell transformation [8].

Little is known about distinct regions of the 708 amino acid long T antigen which are essential for the oligomerization and T-p53 complex formation. It has been reported that phosphorylation of T antigen increases with its oligomerization [9,10]. In our hands, in vitro enzymatic dephosphorylation of T antigen up to 80–90% does not result in the disaggregation of T antigen oligomers [11]. Our recent data indicate different pathways for T antigen oligomerization and T-p53 complex formation [7]. Here, using fragments of large T obtained from different mutants, we found two areas, one within the N-terminal half (114–152) and another area close to the C-terminal end up to amino acid 669 which are essential for oligomerization. Furthermore this truncated C-terminal end at up to amino acid 669 is essential for T-p53 complex formation, whereas the N-terminal region up to amino acid 152 seems to be dispensable.

2. MATERIALS AND METHODS

2.1. *Cells and viruses*

Ad2$^+$D2 virus, originally obtained from G. Fey (University of Lausanne, Switzerland) was grown

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on TC-7 monkey cells as described in [12]. Deletion mutants dl 1066 and dl 1061 were kindly donated by J. Pipas, Pittsburgh, PA, and grown in BSC-40 or COS-7 monkey cells, respectively, as described [13]. COS-7 cells are SV40 transformed monkey cells [14] and CTM cells are mouse cells transformed by CTSV1 [15]. *Pst*I cells, a rat cell line transformed by the SV40 *Pst*I A fragment, were kindly provided by A. Graessman, Berlin, Germany [16]. All cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal calf serum.

2.2. *Infection, radiolabelling and extraction of cells*

Confluent monolayers of TC-7 cells ($3-4 \times 10^6$) were infected with $Ad2^+D2$ for 38 h and with dl 1066 and dl 1061 for 48 h. For radiolabelling virus mutant infected monkey cells or SV40 transformed cells (4×10^6) were washed three times with methionine-free Dulbecco's modified Eagle's medium and then labelled for 2 h with 111×10^4 Bq (30 μCi) [^{35}S]methionine (NEN, Dreieich). After labelling, cells were washed with phosphate-buffered saline, scraped off the plates, pelleted and lysed with 0.4 ml extraction buffer (0.5% Nonidet P40, 10 mM Tris-HCl, pH 9.0, 0.1 M NaCl) [17].

2.3. *Analysis of T antigen and T-p53 complexes*

Fractions of sucrose density gradients or extracts of labelled cells were analysed for T antigen or T-p53 complexes by immunoprecipitation with hamster SV40 tumour sera or monoclonal p53-antibodies, PAb 122. Immunoprecipitates were washed, electrophoresed on 10% SDS-polyacrylamide gels and fluorographed as described in [17,18].

3. RESULTS AND DISCUSSION

In order to study certain N- and C-terminal areas of the polypeptide chain of T antigen which might be necessary for oligomerization we have analysed different fragments of T antigen by sucrose density centrifugation. Monkey cells infected by various SV40 mutants or SV40 transformed monkey, rat or mouse cells were labelled with [^{35}S]methionine for 2 h, extracted and subjected to 5–20% sucrose density gradients as described in [17,18,19]. Fractions of each gradient were immunoprecipitated with hamster SV40 tumour serum and the immunoprecipitates were run on 10% SDS-polyacrylamide gels. Fig.1A shows a typical sedimentation profile of wild-type T antigen from SV40 transformed monkey cells (COS-7) which sediments predominantly in two peaks, one around 5–7 S (mono- and dimers), a second one around 16 S (tetramers) and beyond [4,6]. Fig.1B displays the sedimentation profile of the D2-protein which is a T antigen related hybrid protein encoded by the adenovirus 2-SV40 hybrid virus $Ad2^+D2$ [20]. Instead of the original 114 N-terminal amino acids of T antigen this hybrid protein contains an Ad2 encoded polypeptide (33 kDa) [20]. Thus, this hybrid D2-protein contains amino acids 115–708 from wild-type T antigen. Similarly to intact wild-type T antigen, the D2-protein sediments in low-M_r forms and numerous forms of oligomers sediment down to the bottom of the gradient. According to these data a major N-terminal region ranging from amino acid 1 to 114 appears to be dispensable for the formation of fast sedimenting oligomers of T antigen. This assumption is strongly supported by the observation that aggregates of the D2-protein are typically sensitive to treatment with 20 mM EDTA like wild-type T antigen [18,19].

To amplify further the results obtained with the D2-protein, we selected the deletion mutant CTSV1 [21] which codes for T antigen lacking amino acids 110–152 and overlaps the N-terminal defect of the D2-protein (1–114). To analyse T antigen encoded by CTSV1 for oligomerization we used a CTSV1 transformed mouse cell line CTM [15]. As seen in fig.1C this deleted T antigen sediments predominantly between 5 and 14 S and fails to aggregate to 16 S and higher oligomers. Thus, the region between amino acid 110 and 152 including the N-terminal phosphorylation sites Ser 111, Ser 123 and Thr 124 [3] is obviously essential for the formation of 16 S and higher oligomers.

To investigate the C-terminal region of T antigen for oligomerization we selected from a few deletion mutants known to be viable, the dl 1066 mutant [13] and, additionally dl 1061 as a non-viable example [13]. Extracts from monkey cells infected by dl 1066 or dl 1061 were analysed on sucrose density gradients. The profile of dl 1066 T antigen missing C-terminal amino acids 670–708

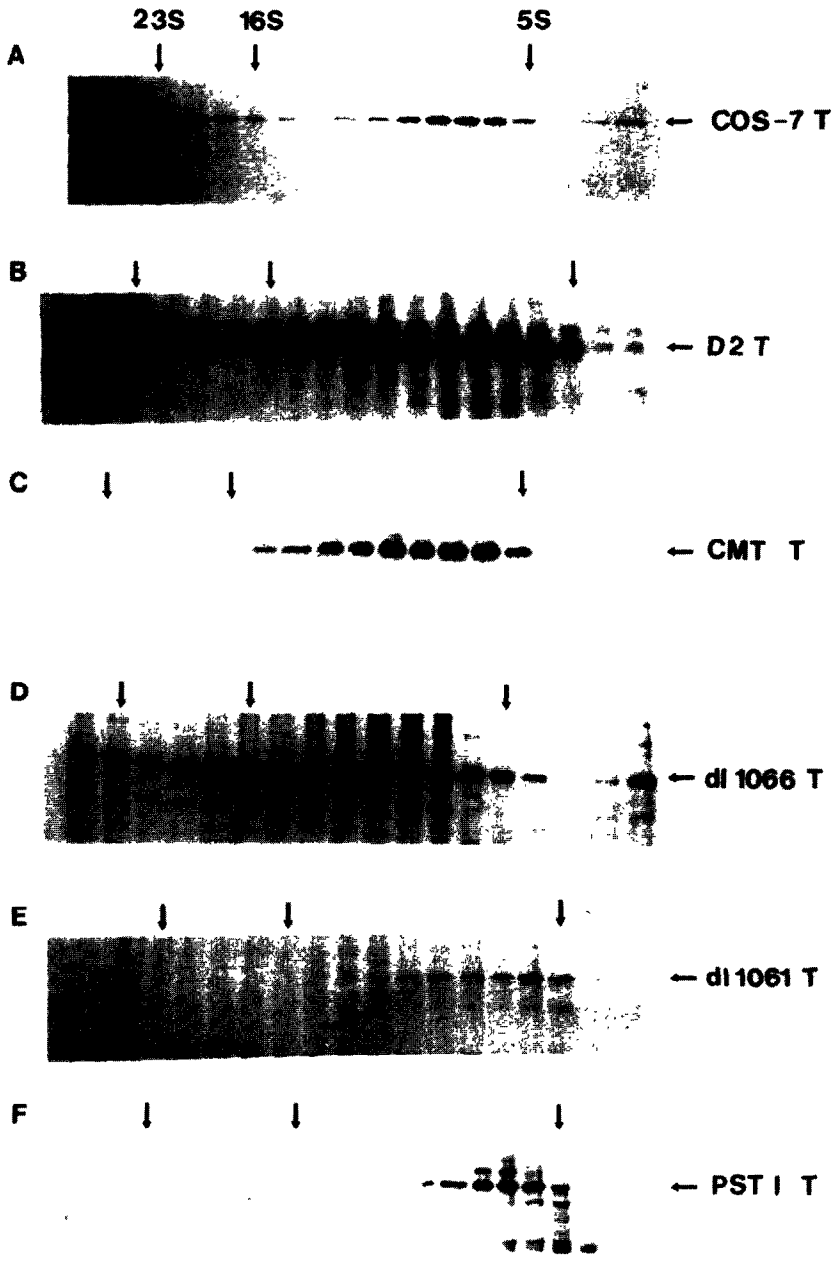

Fig.1. Sedimentation profiles of T antigen characterized by various deletions in N- and C-terminal regions. Cells were labelled with [^{35}S]methionine and extracts were analysed by 5–20% sucrose density gradient centrifugation. Fractions were immunoprecipitated with hamster SV40 tumour serum, run on 10% SDS-polyacrylamide gels and fluorographed as described [17]. Markers for gradient centrifugation were rRNAs (5 S, 16 S and 23 S). Only the sedimentation profiles of individual T antigen fragments are shown: (A) wild-type T antigen from COS-7 cells, M_r 90000; (B) D2-protein from Ad2$^+$D2 infected monkey cells (TC-7), M_r 107000; Ad 120K protein [12] is detected above the D2-T; (C) T antigen lacking amino acids 110–152 from CTSV1-transformed mouse cells CTM, M_r 83000; (D) truncated dl 1066 encoded T antigen from infected TC-7 cells (amino acids 1–669), M_r 87000; (E) dl 1061 T antigen, M_r 77000, analysed like (D); (F) truncated T antigen from *Pst*I A transformed rat cells (amino acids 1–538), M_r 80000. For details see section 2.

(fig.1D) presents low- and high-M_r forms which sediment in a broad distribution over the entire gradient. Aggregates sedimenting around 16 S and beyond are sensitive to EDTA treatment like the D2-protein and wild-type T antigen (not shown) [18,19]. This suggests that dl 1066 T antigen fulfills requirements for native oligomerization of wild-type T antigen. In contrast, T antigen encoded by the nonviable dl 1061 mutant, lacking amino acids 591–708, fails to oligomerize to 16 S and higher aggregated forms (fig.1E) and the profile is not influenced by treatment with EDTA (not shown). An even shorter truncated T antigen from rat cells transformed by the *Pst*I A fragment of SV40 DNA [16] lacking amino acids 539–708 is also unable to oligomerize (fig.1F) like dl 1061 T antigen.

According to these results we can conclude that the native C-terminal area between amino acid 669 and 708 is not involved in oligomerization. However, extension of this truncation upstream to amino acid 591 destroys this function. Consequently the phosphorylated residues Ser 676, Ser 677, Ser 679 and Thr 701 [3] are also negligible for this mechanism. Taking these results together, one can postulate that both a C-terminal region between amino acid 591 and 669 and an N-terminal region in the second exon between amino acid 115 and 152 are essential for the formation of highly oligomeric forms.

In previous experiments, we and others have shown that in SV40 infected and transformed cells, p53 is mostly found in high-M_r T-p53 complexes [8,9]. One might speculate that the oligomerization of T antigen may be somehow involved in the process of T-p53 complex formation. Therefore we addressed the question of whether a truncated T antigen which is unable to aggregate into 16 S oligomers and higher aggregates might also be defective in binding to p53. We tested the oligomerization-defective dl 1061 T antigen and, as a control, again the dl 1066 T antigen for the formation of T-p53. On the other hand, since the D2-protein missing amino acids 1–114 is able to bind p53 [22] we analysed CTSV1 T antigen lacking amino acids 110–152 for complexing p53. Cells

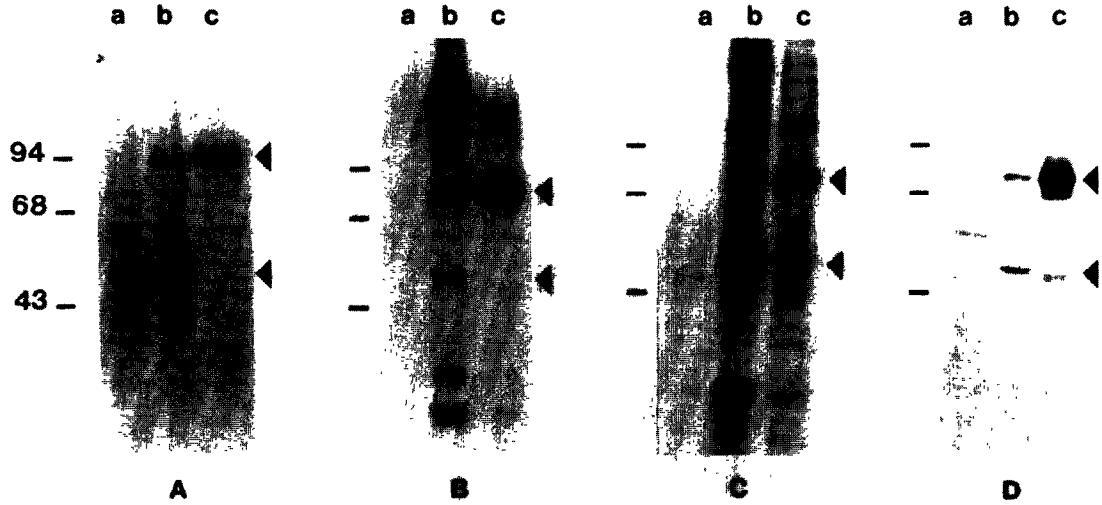

Fig.2. Sequential immunoprecipitation of T-p53 complexes and free T antigen from COS-7 cells (A), dl 1066 (B) and dl 1061 (C) infected monkey cells and CTSV1 transformed mouse CTM cells (D). In each case approx. 4×10^6 cells were labelled with [^{35}S]methionine and cell extracts were incubated first with normal hamster serum (lane a) followed by PAb 122 (lane b) to precipitate p53 and T-p53 complexes. The final supernatants were analysed for free T antigen by incubation with hamster SV40 tumour serum (lane c). Immunoprecipitates were electrophoresed and fluorographed on 10% SDS-polyacrylamide gels. Markers are indicated on the left: phosphorylase *a* (94 kDa), bovine serum albumin (68 kDa) and ovalbumin (43 kDa). T antigen and p53 are indicated by arrowheads on the right.

were labelled with [^{35}S]methionine for 2 h and lysed as described in section 2. To detect T-p53 complexes, extracts were first treated with the monoclonal antibody PAb 122 directed against p53 [23]. Free T antigen was immunoprecipitated from the supernatant with hamster SV40 tumour serum. The controls, native T-p53 complexes (lane b) and free T antigen (lane c) from COS-7 cells are shown in fig.2A. As expected, T antigen encoded by viable dl 1066 forms stable complexes with p53 (fig.2B, lane b) whereas dl 1061 T antigen which is defective for oligomerization is also unable to bind p53 (fig.2C, lane b). In contrast, we detected p53 stably complexed with the N-terminally deleted CTSV1 T antigen in extracts of CTM cells (fig.2D, lane b). In all cases considerable amounts of free T antigen were found in the supernatants (fig.2A–D, lanes c).

The combined data obtained with the D2-protein and CTSV1 T antigen show that a major N-terminal region of amino acids 1–152 is not critical for T-p53 complex formation. Since CTSV1 T antigen from CTM cells is defective for the formation of tetramers (16 S) and higher oligomers this process can be excluded as a prerequisite for T-p53 complex formation. Furthermore, this observation confirms our recent results showing that T antigen encoded by tsA1499 is heat-sensitive for oligomerization but not for complex formation with p53 [7,8]. The deletion of this mutant from amino acid 635 to 661 includes phosphorylated Ser 639 [3,24]. Since tsA1499 T antigen can oligomerize into high-M_r aggregates and complex p53 [7,8] we can narrow down the above-mentioned C-terminal (591–669) region necessary for oligomerization and T-p53 complex formation to amino acids 591–634. According to these deletions, we can exclude all five known C-terminal phosphorylation sites as having a functional role in oligomerization as well as in T-p53 complex formation. The potential influence of individual N-terminal phosphorylation sites on the oligomerization of T antigen needs to be determined.

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