SIMIAN VIRUS 40 T-ANTIGEN PHOSPHORYLATION IS VARIABLE

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1. Introduction

Simian virus 40 (SV40)-transformed tumor cells contain at least two SV40 coded proteins: Little t- (MW 21 000) and large T-antigen (MW 94 000). Large T-antigen (T-Ag) is located in the cell nucleus [1] and seems to be involved in the synthesis of viral DNA and RNA in permissive cells, as well as of host cell DNA and RNA in transformed cells. Furthermore, this protein is apparently responsible for the initiation and the maintenance of cell transformation (review in [2]). Besides the amino acid sequence of T-Ag which can be derived from the known nucleotide sequence of SV40 DNA [3,4], only a few biochemical properties of T-Ag are known: (i) In 1977 P. Tegtmeyer reported that T-Ag is a phosphoprotein [5]; (ii) T-Ag has a nonspecific binding affinity to double-stranded (ds) and single stranded (ss) DNA [6–8] and a specific binding affinity to the region of the origin of replication of SV40 DNA [7,9,10]. This report describes that the phosphorylation of T-Ag is variable and it gives a preliminary characterization of an apparently positive correlation between the phosphorylation and the binding affinity of T-Ag to calf thymus ds-DNA.

2. Materials and methods

2.1. *Labelling of cells*

SV40 T-Ag was isolated from [^{3}H]leucine/[^{32}P]-phosphate-labelled extracts of SV40 transformed human cells (SV80) by indirect immunoprecipitation using hamster SV40 tumor serum. 5×10^6 cells grown in a monolayer in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 5% fetal calf serum were labelled in 1 ml of leucine and phosphate deficient DMEM containing 20 μCi [^{3}H]leucine and 100 μCi [^{32}P]phosphoric acid for 2 h at 37°C. The harvested cells were washed with PBS and lysed with 0.5% NP40 in 0.01 M Tris · HCl pH 9.0, 0.1 M NaCl, for 30 min on ice and then centrifuged at 105 000 × g for 30 min. This supernatant is called 'cell extract'. In control experiments cells were labelled up to 24 h simultaneously with [^{3}H]leucine and [^{32}P]phosphate without any significant influence on the results described below.

2.2. *Incubation of cell extracts with DNA-cellulose*

0.4 ml aliquots of the cell extract were incubated either directly with 30 mg calf thymus DNA cellulose (P. L. Biochemicals) or after adjustment to pH 6.0 or 7.3 with 0.1 M acetic acid. After shaking for 30 min at 4°C the DNA cellulose was spun down washed once with 0.4 ml of the same buffer at the identical pH. The supernatants were collected and analyzed for T-Ag. The DNA cellulose was eluted twice with 0.4 ml 0.01 M Tris · HCl pH 9.0, 0.8 M NaCl containing 0.5% NP40 and the combined eluates were analyzed for T-Ag. Treatment with alkaline phosphatase: The ^{3}H/^{32}P-labelled cell extracts were incubated for 60 min at 22°C with 5 units/ml alkaline phosphatase (Boehringer, FRG).

Trichloroacetic acid precipitates: 10 μl of the supernatants or eluates of the DNA cellulose incubation were made 10% in trichloroacetic (TCA) in the presence of 1 mg bovine serum albumin as carrier protein. The precipitates were washed intensively with cold 10% TCA containing 0.5 M cold sodium phosphate and 0.5 M leucine. The precipitates were counted for ^{3}H- and ^{32}P-radioactivities in Quickszint 402 (Zinsser).

2.3. *Isolation of SV40 T-Ag by immunoprecipitation*

Unspecific proteins were removed from the DNA cellulose supernatants and eluates by preprecipitation

using 20 μl normal hamster serum and 200 μl of a 10% suspension of *Staphylococcus aureus* (strain Cowan I) as described by Kessler [11]. After 4 h incubation at 4°C *S. aureus* were spun down. T-Ag was precipitated from this supernatant by incubation with 20 μl hamster SV40 tumor serum and 200 μl *S. aureus* overnight at 4°C. The immunoprecipitates were washed 5 times with 0.15 M NaCl, 5 mM EDTA, 50 mM Tris · HCl pH 7.4 (NET-buffer) containing 0.5% NP40. Hamster SV40 tumor sera with high titers against SV40 T-Ag were obtained from Syrian Gold Hamsters 3–6 weeks after a subcutaneous injection with 2 × 10^6 SV40 transformed hamster cells (H 65/90 B).

2.4. *Analysis of SV40 T-Ag phosphorylation*

T-Ag was eluted by boiling the immunoprecipitates for 10 min in SDS-sample buffer (2% SDS, 5% mercaptoethanol, 65 mM Tris · HCl pH 6.8, 0.01% bromophenol blue) and run on 10 cm discontinuous 8.5% SDS polyacrylamide gels (PAGE) according to Läemmli [12]. After electrophoresis the gels were frozen and cut into 2 mm slices which were eluted overnight with 200 μl water and counted for ^{3}H- and ^{32}P-radioactivity in Quickszint 402.

3. Results and discussion

Fig.1 shows a SDS-polyacrylamide gel with SV40 T-Ag isolated by indirect immunoprecipitation from 0.5% NP40/pH 9.0 extracts of [^{3}H]leucine/[^{32}P]-phosphate-labelled SV40 transformed human cells (SV80). To judge the experiments described below, it was particularly important to demonstrate that the recognition of T-Ag by antibodies is not influenced by the degree of phosphorylation. Treatment of a ^{3}H/^{32}P-labelled cell extract with alkaline phosphatase reduced the phosphate content of T-Ag by 20–50% without changing the yield of T-Ag.

To analyze whether the phosphorylation of T-Ag is constant or variable we compared the ^{3}H/^{32}P-ratios of T-Ag in the supernatants after incubation of extracts of [^{3}H]leucine/[^{32}P]phosphate-labelled SV80 cells with calf thymus DNA-cellulose at pH 6.0, 7.3 and 9.0 (table 1). One can see a significant variation in the phosphate content of T-Ag from DNA cellulose supernatants obtained at different pHs indicating that T-Ag exists in different phosphorylated forms. Additionally, the affinity of T-Ag to DNA is strongly increasing with acid pH.

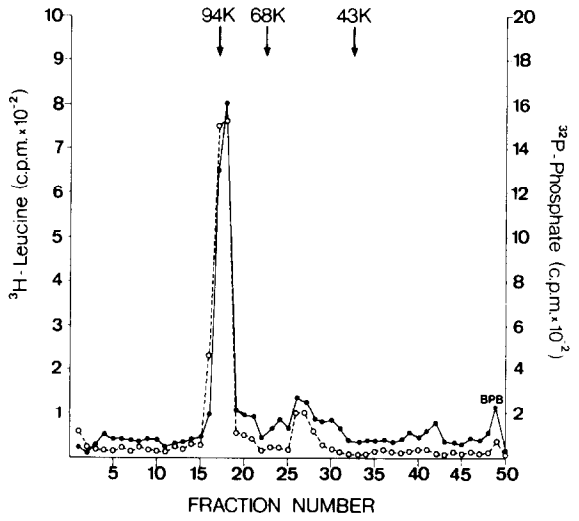

Fig.1. SDS-PAGE of T-Ag immunoprecipitated from NP40 extracts of SV80 cells labelled with [^{3}H]leucine and [^{32}P]-phosphate. MW markers: Phosphorylase A (94 000), bovine serum albumin (68 000), ovalbumin (43 000), BPB, bromophenol blue, ^{3}H (•——•), ^{32}P (○– – –○).

These indirect results obtained in many experiments suggest that in addition to the strong but unspecific influence of the pH, the affinity of T-Ag to DNA seems to be increased by phosphorylation. This hypothesis was tested by two direct approaches: (i) Analysis of the ^{3}H/^{32}P-ratio of T-Ag eluted from DNA-cellulose and direct comparison with T-Ag which did not bind under these conditions; (ii) investigation of the influence of a partial enzymatic dephosphorylation on the affinity of T-Ag to DNA.

Table 1
Binding of SV40 T-Ag to calf thymus DNA-cellulose: influence of T-Ag-phosphorylation and pH

pH	Supernatant cpm			Eluate cpm		
	^{3}H	^{32}P	^{3}H:^{32}P	^{3}H	^{32}P	^{3}H:^{32}P
6.0	182	530	1:2.91	528	2445	1:4.63
7.3	588	2058	1:3.50	143	631	1:4.41
9.0	636	2011	1:3.16	105	650	1:6.20

Comparison of T-Ag immunoprecipitated from the supernatant and from eluates of DNA cellulose. The radioactivities of T-Ag were obtained from ^{3}H and ^{32}P-cpm from at most two gel slices in the T-Ag peak. The T-Ag peak of the immunoprecipitate from the original SV80 cell extract contained 732 cpm ^{3}H, 2380 cpm ^{32}P, ^{3}H/^{32}P ratio 1:3.25

Table 2
Binding of total proteins in extracts to calf thymus DNA cellulose: influence of phosphorylation and pH

pH	Supernatant cpm			Eluate cpm		
	^3H	^{32}P	^3H:^{32}P	^3H	^{32}P	^3H:^{32}P
6.0	121 753	425 226	1:3.49	75 845	150 605	1:1.985
7.3	181 930	535 229	1:2.85	34 766	77 606	1:2.23
9.0	192 966	547 423	1:2.85	49 100	91 805	1:1.87

Binding of $^3H/^{32}P$-labelled total cell proteins to calf thymus DNA cellulose. Comparison of the phosphorylation of the total proteins in the trichloroacetic acid precipitates from DNA cellulose supernatants and eluates. $^3H/^{32}P$ ratio in the trichloroacetic acid precipitate from the original SV80 cell extract: 1:2.96

The results of the first approach are shown in table 1. T-Ag bound to DNA-cellulose at pH 6.0, 7.3 or 9.0 was eluted at pH 9.0 in the presence of 0.8 M NaCl and subsequently analyzed by immunoprecipitation followed by SDS-PAGE. According to the $^3H/^{32}P$-ratios the T-Ag molecules eluted from DNA contain between 1.2 and 2.0 times more [^{32}P]phosphate than unbound T-Ag molecules in the corresponding supernatants.

This apparent correlation between the degree of phosphorylation and the DNA binding affinity seems to be a peculiar property of T-Ag when compared with the DNA binding behaviour of the total proteins in the extract under identical conditions (table 2). This was assayed by measuring the ^3H- and ^{32}P-radioactivities in 10% TCA protein precipitates of total SV80 cell extracts of DNA cellulose supernatants at different pHs and of the corresponding DNA-cellulose pH 9.0/0.8 M NaCl eluates. The results demonstrate clearly two remarkable differences in comparison to T-Ag (table 1): (i) The proteins eluted from DNA-cellulose in each case contain less phosphate than the proteins in the corresponding supernatant; (ii) at pH 6.0 the total proteins do not bind as strongly to DNA as T-Ag.

According to these results a partial enzymatic dephosphorylation of T-Ag should decrease its binding affinity to DNA. This is demonstrated in table 3. [^3H]Leucine/[^{32}P]phosphate-labelled SV80 cell extracts were incubated with DNA-cellulose under identical conditions as used for tables 1 and 2 but with or without alkaline phosphatase treatment. In this experiment T-Ag was dephosphorylated by ~45% ($^3H/^{32}P$ = 1:1.82) when compared with T-Ag

Table 3
Influence of partial dephosphorylation of T-Ag by alkaline phosphatase on its affinity to calf thymus DNA cellulose

SV80 cell extract	T-Ag in supernatants after DNA-cellulose incubation								
	pH 6.0 cpm			pH 7.3 cpm			pH 9.0 cpm		
	^3H	^{32}P	$^3H/^{32}P$	^3H	^{32}P	$^3H/^{32}P$	^3H	^{32}P	$^3H/^{32}P$
Without treatment	104	212	1:2.04	551	2050	1:3.72	609	2201	1:3.61
With alkaline phosphatase treatment	250	183	1:0.72	793	1118	1:1.41	685	871	1:1.27

An SV80 cell extract was incubated with DNA cellulose at pH 6.0, 7.3 and 9.0 as described in Section 2 either before or after treatment with alkaline phosphatase. T-Ag was immunoprecipitated from the supernatants and analyzed by SDS-PAGE. $^3H/^{32}P$ ratio in the T-Ag peak of an immunoprecipitate from the original SV80 cell extract: (a) untreated 1:3.25 (b) treated with alkaline phosphatase 1:1.82

in the untreated cell extract ($^3H/^{32}P$ = 1:3.25). Partial dephosphorylation of T-Ag in the total cell extract decreased the binding rate of T-Ag to DNA by a factor of ~2.5 at pH 6.0, 1.5 at pH 7.3 and 1.25 at pH 9.0 as calculated from the ^3H cpm shown in table 3. These data confirm the previous results and support the hypothesis that the dephosphorylation can be correlated with a decrease in the affinity of T-Ag to DNA very strongly at pH 6.0 but also at pH 7.3 and weakly at pH 9.0.

From these results one can conclude that (i) SV80 cell extracts contain two or more forms of T-Ag differing in their degree of phosphorylation. Recently it was reported that the phosphate groups of T-Ag are apparently turned over at two different rates, both faster than the lifetime of the protein [13]. (ii) Different T-Ag can be separated according to their affinity for DNA. (iii) The affinity of T-Ag for DNA cellulose in a total cell extract in addition to the unspecific binding at acid pH seems to increase with its phosphorylation. In this respect T-Ag can be compared with histones as well as some non-histone DNA and RNA-binding proteins whose DNA binding affinity can be controlled by phosphorylation [14,15]. We analyzed the binding of T-Ag to DNA in cell extracts containing many proteins and other factors which could influence or even mediate the binding of T-Ag to DNA. A possible contribution of 'middle T' [16,17] seen in fig.1 (fractions 24–26, MW 50 000–55 000) can at the moment under our experimental conditions neither be determined nor excluded. Further studies using purified T-Ag and different nucleic acids should allow to characterize this type of protein-nucleic acid interaction in more detail.

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