Relationship of phosphorylation to the oligomerization of SV40 T antigen and its association with p53

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The potential significance of the phosphorylation of SV40 large T antigen for oligomers and complexes with the cellular protein p53 was investigated. We observed that T antigen oligomers remain stable after enzymatic dephosphorylation by alkaline phosphatase up to 80%. Separate analysis of free and p53-bound T antigen revealed a considerably lower phosphorylation of the p53-bound subclass. Therefore, a simple correlation between the overall phosphorylation of T antigen and the formation of oligomers and T-p53 complexes is highly unlikely.

SV40 large T antigen *Oligomerization* *Phosphorylation* *T antigen-p53 complex* *Alkaline phosphatase*

1. INTRODUCTION

Simian virus 40 (SV40) large T antigen is a regulatory protein which plays an essential role in virus growth and cell transformation (for references see [1]). This protein has a molecular mass of approx. 82 kDa and is phosphorylated at up to 11 sites which are localized mainly in two separate clusters on the polypeptide chain [2–4]. This extremely variable modification was reported to be involved in the conversion of T antigen into its variable quaternary structures [5–7] and to influence functional aspects such as its subcellular localization [8] and its interaction with cellular [9] and SV40 DNA [10, 11].

Various homologous oligomeric forms of T antigen and, additionally, heterologous oligomers which are composed of stable complexes of T antigen with the cellular p53 protein [6, 12] arise by self-assembly after posttranslational modification of newly synthesized monomers [5, 12–15]. According to several previous reports, this processing seems to be generally correlated with an increase in the phosphorylation of T antigen, suggesting that the overall phosphorylation may be principally essential for oligomerization and T-p53 complex formation [6, 7, 12, 13]. However, this conclusion is questionable as it has not been satisfactorily proven: first, in none of these experiments were p53 bound and unbound forms of T antigen analyzed separately for specific phosphorylation. Second, Prives and co-workers [10] detected no distinct differences between the specific phosphorylation of various oligomeric forms. Therefore, we analyzed the sedimentation profiles of T antigen on sucrose density gradients with and without prior enzymatic dephosphorylation. Furthermore, we determined the specific phosphorylation of p53-bound and unbound forms of T antigen.

2. MATERIALS AND METHODS

2.1. *Cells, labeling conditions and extraction of cells*

SV80, and SV40 transformed human cell line, was routinely grown in Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal calf

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serum. 4×10^6 cells were labeled either with 30 μCi [^{35}S]methionine, 100 μCi [^{32}P]phosphate or 100 μCi [^{32}P]phosphate and 20 μCi [^{3}H]leucine in methionine-, phosphate- or phosphate- and leucine-free DMEM. Cells were washed and lysed as in [15].

2.2. *Enzymatic dephosphorylation, sucrose density gradient centrifugation, immunoprecipitation and SDS-polyacrylamide gel electrophoresis*

Clarified cell extracts (400 μl, 8×10^6 cells) were dialyzed for 14 h at 4°C with or without 30 units alkaline phosphatase (Boehringer, Mannheim) against 10 mM Tris-HCl, pH 8.3, 5 mM $MgCl_2$, 0.5% NP40 and then run on 5–20% sucrose gradients for 14 h at 36 000 rpm in a Beckman SW41 rotor. Fractionation of the gradient, immunoprecipitation and SDS-polyacrylamide gel electrophoresis were performed as in [15].

2.3. *Determination of the specific phosphorylation of T antigen*

For the determination of the specific phosphorylation of T antigen, gels were stained with Coomassie blue and the T antigen bands were scanned with a Quick scan densitometer R & D, Helena Laboratories, excised from the gel and counted for ^{32}P radioactivity in a Packard β-counter by Čerenkov radiation. Specific phosphorylation was estimated by the ratio of ^{32}P cpm per integration unit obtained by densitometric scanning.

3. RESULTS AND DISCUSSION

We first investigated the possible functional role of the total phosphorylation of T antigen on the stability of oligomers by enzymatic in vitro dephosphorylation. Extracts (0.4 ml) of 4 h ^{32}P-labeled SV80 cells adjusted to pH 8.3 and to 5 mM $MgCl_2$ were dialyzed at 4°C for 14 h in the presence or absence of 30 units alkaline phosphatase. T antigen was immunoprecipitated from both extracts with hamster SV40 tumor serum and the specific phosphorylation was determined by densitometric scanning of Coomassie blue-stained T antigen bands and counting the ^{32}P radioactivity in these bands. Reproducibly, under these optimized conditions T antigen was dephosphorylated by 65–80% (not shown). To analyze the oligomerization pattern of T antigen under these conditions, extracts of [^{35}S]methionine-labeled SV80 cells were incubated with or without alkaline phosphatase and run on 5–20% sucrose density gradients. As shown in fig. 1A and B, this major dephosphorylation causes no distinct change in the sedimentation profile of T antigen. This observation suggests that not the majority, but only very few phosphate groups may be involved in the protein-protein interactions between oligomers of T antigen.

To determine the specific phosphorylation of p53-bound and unbound T antigen SV80 cells were radiolabeled with [^{32}P]phosphate and lysed as in [15]. Cell extracts were first incubated with a monoclonal antibody directed against p53 (PAb 122; for references see [16] to precipitate T-p53 complexes. The supernatants were subsequently immunoprecipitated with hamster SV40 tumor serum to obtain free T antigen. The immunoprecipitates were analyzed on 10% SDS-polyacrylamide gels [17]. The gels were stained with Coomassie blue, dried and autoradiographed for ^{32}P radioactivity. As shown in fig. 2 PAb 122 antibodies precipitated p53 complexed with a considerable amount of T antigen (lane M). A large amount of free T antigen but no p53 was brought down by hamster SV40 tumor serum (lane T).

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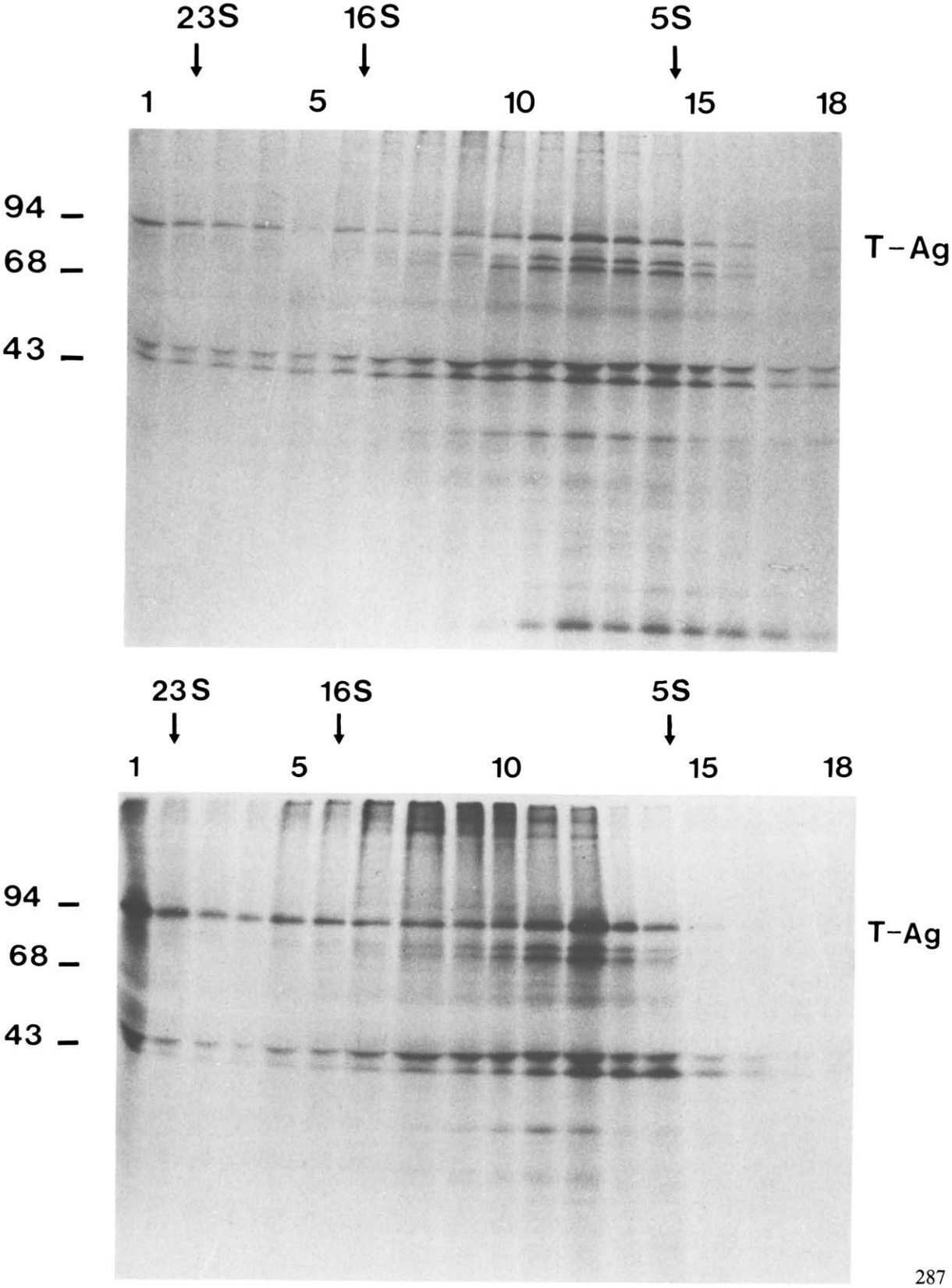
Fig. 1. Sucrose density gradient centrifugation of T antigen from SV80 cells before and after extensive alkaline phosphatase treatment. 0.4 ml extracts of 8×10^6 [^{35}S]methionine-labeled SV80 cells were incubated with (A) and without (B) 30 units alkaline phosphatase for 14 h at 4°C and analyzed by sucrose density gradient centrifugation (5–20%). Fractions of gradients were immunoprecipitated with hamster SV40 tumor serum, electrophoresed on 10% SDS-polyacrylamide gels and fluorographed. M_r markers for sucrose gradient centrifugation were rRNAs (5 S, 16 S and 23 S), for SDS-polyacrylamide gel electrophoresis; M_r markers were phosphorylase *a*, M_r 94 000; bovine serum albumin, M_r 68 000; ovalbumin, M_r 43 000).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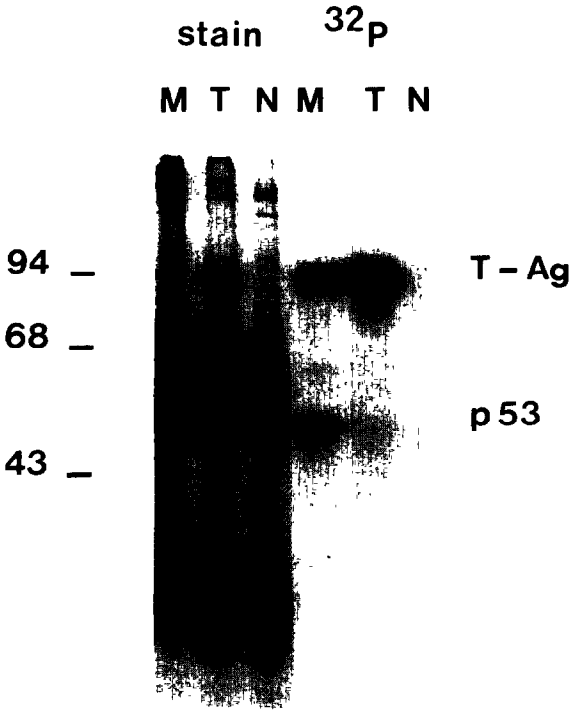

Fig. 2. Sequential immunoprecipitation of T antigen-p53 complexes and free T antigen from SV80 cells. An extract (0.4 ml) of [^{32}P]phosphate-labeled SV80 cells were treated with PAb 122 (M) and subsequently with hamster SV40 tumor serum (T). (N) Normal hamster serum control immunoprecipitates. Immunoprecipitates were analyzed on a 10% SDS-polyacrylamide gel. The gel was stained with Coomassie blue (3 lanes on the left), dried and exposed for autoradiography (3 lanes on the right).

Control experiments using normal hamster serum gave neither Coomassie blue stained nor ^{32}P-labeled bands corresponding to T antigen or p53 (lane N). To exclude time-dependent ^{32}P incorporation into certain subclasses of T antigen, cells were labeled for various times (2, 4, 15, 24 h). Coomassie blue-stained T antigen bands were quantitated by densitometer tracing and the incorporation of [^{32}P]phosphate was measured by excising these bands and counting for ^{32}P cpm. The ratios of the specific phosphorylation of p53-bound to unbound T antigen listed in table 1

Table 1

Comparative analysis of the specific phosphorylation of p53-bound and unbound populations of T antigen from SV80 cells

	^{32}P labeling time							
	2 h		4 h		15 h		24 h	
	p53-bound T	Unbound T	p53-bound T	Unbound T	p53-bound T	Unbound T	p53-bound T	Unbound T
Densitometry of Coomassie blue-stained T bands (i.u.)	44	222	49	342	45	84	43	174
^{32}P cpm in excised gel bands	189	1540	72	1236	389	1388	355	2487
Specific radioactivity (^{32}P cpm/i.u.)	4.30	6.49	1.47	3.61	8.64	16.52	8.26	14.29
Ratio of specific radioactivities of p53-bound T: free T	0.62		0.41		0.52		0.58	

Comparative analysis of the specific phosphorylation of p53-bound and unbound T antigen from SV80 cells. 0.4 ml extracts of [^{32}P]phosphate-labeled SV80 cells were treated with PAb 122 and subsequently with hamster SV40 tumor serum. Immunoprecipitates were analyzed on a 10% SDS-polyacrylamide gel. The gel was stained with Coomassie blue, dried and exposed for autoradiography. The specific phosphorylation of each T antigen band is expressed as the ratio of ^{32}P cpm/integration unit (i.u.) obtained by densitometer tracing. The direct correlation between protein concentration and densitometer scanning was assayed routinely by scanning various amounts of a standard solution of phosphorylase (M_r 94 000)

vary at different labeling times by factors between 0.41 and 0.62, but indicate clearly that under all labeling conditions p53-bound T antigen was considerably less phosphorylated than unbound T antigen.

We applied the same method using SV40-adenovirus 7 hybrid virus transformed hamster cells (kindly donated by J. Butel, Houston, USA), which express predominantly cytoplasmic (J19L) or nuclear T antigen (8L [8]) and obtained similar results. In all cases T antigen bound to p53 was 23–60% less phosphorylated than free T antigen. In agreement with previous results [8] cytoplasmic T antigen was less phosphorylated than nuclear T antigen (not shown).

In a second approach the phosphorylation of p53-bound and unbound T antigen was examined by double labeling SV40-transformed human (SV80) or rat cells (SV7 [18]) with [^{3}H]leucine and [^{32}P]phosphate for 4 h, a method which has been applied by several laboratories [4, 6, 9]. p53-bound and unbound T antigen forms were separated by differential precipitation from cell extracts as described above. As shown in table 2, in both cell lines p53-bound T antigen was approx. 30% less phosphorylated than the unbound population of large T. These data confirm the results obtained with SV80 cells (table 1) which can now be extended to include SV40-transformed rat cells. In summary, in SV40-transformed human, rat and hamster cells, p53 bound T antigen is distinctly less phosphorylated than free T antigen.

So far, no molecular details about the mechanism of the conversion of T antigen into oligomers and the formation of T-p53 complexes are known. Statements about the phosphorylation of T antigen in different oligomeric forms [5–7] are questionable because nobody has separately analyzed the phosphorylation of free and p53 bound T antigen. As shown in this study both T antigen forms are quite differently phosphorylated. Levine and co-workers [19] reported a similar observation about the E1b 58 K protein from adenovirus 5 transformed C3H cells which is also less phosphorylated in the complex with p53 than in the free form. It is still unclear whether a low but distinct phosphorylation is essential for T-p53 complex formation or, alternatively, whether T antigen associated with p53 is more efficiently dephosphorylated than free T antigen.

Confirming and extending the above mentioned statements [5–7], very recently Scheidtmann et al. [11] found that newly synthesized mainly monomeric large T antigen was in a low phosphorylation state whereas older T antigen predominantly in oligomeric forms was higher phosphorylated. Since both subclasses exhibit a distinct phosphorylation pattern shown by two-dimensional peptide mapping one has to assume that only distinct phosphorylation sites are involved in the process of oligomerization [11]. As estimated from our present data only 1–2 if any of the phosphate sites of T antigen are involved in the protein-protein interaction between subunits of oligomers of T antigen. By analyzing the

Table 2

Phosphorylation of p53-bound and unbound T antigen in [^{3}H]leucine and [^{32}P]phosphate double-labeled SV80 or SV7 cells

Cells	p53-bound T antigen			Unbound T antigen			^{32}P/^{3}H ratio p53-bound vs unbound T antigen
	^{3}H cpm	^{32}P cpm	^{32}P/^{3}H ratio	^{3}H cpm	^{32}P cpm	^{32}P/^{3}H ratio	
SV80	705	860	1.22	1511	2587	1.71	0.71
SV7	622	957	1.54	2298	5139	2.24	0.69

Phosphorylation of p53-bound and unbound T antigen in [^{3}H]leucine and [^{32}P]phosphate double-labeled SV80 or SV7 cells. Extracts (0.4 ml) of [^{3}H]leucine and [^{32}P]phosphate double-labeled SV80 or SV7 cells were treated with PAb 122 and subsequently with hamster SV40 tumor serum. The immunoprecipitates were analyzed on a 10% SDS-polyacrylamide gel. The radioactivities in the T antigen bands were determined by excising the bands and counting for ^{3}H and ^{32}P radioactivity. The phosphorylation of each T antigen band is expressed as the ratio of ^{32}P cpm vs ^{3}H cpm.

oligomerization properties of T antigen encoded by deletion mutants and several C- and N-terminal fragments of T antigen we could exclude an influence of the C-terminal phosphorylation sites on the oligomerization of T antigen (to be published).

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