CHARGE ISOMERS OF SIMIAN VIRUS 40 T-ANTIGEN

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Received 24 July 1980

1. Introduction

The genome of Simian Virus 40 (SV40), a small DNA containing tumor virus, codes for at least two non-structural proteins (T- and t-antigen) which are expressed in permissive monkey cells early after virus infection as well as in SV40 transformed cells. There is considerable evidence that T-antigen (T-Ag) is required for:

(i) The initiation of SV40 DNA replication, as well as the stimulation of cellular DNA synthesis;
(ii) The transcription of the late SV40 genes;
(iii) The regulation of its own synthesis; and
(iv) The establishment and maintenance of transformation (review [1]).

In contrast to these important biological observations, little is known about detailed biochemical properties of T-Ag as a phosphoprotein with M_r 94 000 [2]. Since many cellular enzymes are known to be regulated by post-translational modification of the polypeptide chain [3], phosphorylation of T-Ag might be one mechanism to modulate the activity of T-Ag. Charge variations may be detected and analyzed by the high resolving power of isoelectric focusing (IEF). First attempts [4] to separate differently charged T-Ag molecules by 2-dimensional gel techniques showed that T-Ag does not form discrete spots, but rather forms a broad streak indicating some unusual isoelectric focusing properties of T-Ag. In addition sodium dodecylsulfate gel electrophoresis (SDS–PAGE)-purified T-Ag analyzed by isoelectric focusing does not show a stable charge pattern [5].

This study shows that T-Ag exists as two charge isomers. After purification of T-Ag by SDS–PAGE [^{35}S]methionine- or $^{32}PO_4$-labeled T-Ag focuses at pH ~6.5 in two stable bands. No unphosphorylated charge isomers of T-Ag could be observed.

2. Materials and methods

2.1. *Cell culture and immunoprecipitation*

SV40 T-Ag was isolated from extracts of either [^{35}S]methionine- or $^{32}PO_4$-labeled SV40-transformed human cells (SV80) by indirect immunoprecipitation using hamster SV40 tumor serum obtained from Syrian Gold hamsters 3–6 weeks after a subcutaneous injection with 2×10^6 SV40-transformed hamster cells (H 65/90 B) [6]. About 5×10^6 SV80 cells grown in a monolayer in Dulbecco's Modified Eagle's Medium (DMEM) containing 5% fetal calf serum were labeled for 8 h at 37°C in 1 ml methionine- or phosphate-deficient DMEM containing 50 μCi [^{35}S]methionine (Amersham) or 100 μCi [^{32}P]phosphate (NEN). The cells were washed and lysed for 30 min on ice in 0.1 M Tris–HCl (pH 9.0), 0.1 M NaCl, 0.5 mM $MgCl_2$ containing 0.5% of the non-ionic detergent Nonidet P40 (NP40, Fluka) and 1% Trasylol (Bayer). The lysate was centrifuged at 105 000 × *g* for 30 min. After a preprecipitation of this supernatant with 20 μl normal goat serum and 200 μl 10% *Staphylococcus aureus* suspension strain Cowan I (1 h, 4°C) T-Ag was immunoprecipitated from the supernatant by adding 20 μl hamster SV40 tumor serum (30 min, 4°C) and 200 μl *S. aureus* (overnight at 4°C) as in [7]. After washing the immunoprecipitates with 0.15 M NaCl, 5 mM EDTA, 50 mM Tris–HCl (pH 7.4) and 1% sucrose containing 1% NP40, T-Ag was eluted by boiling the immunoprecipitates for 3 min in sample buffer (65 mM Tris–HCl (pH 7.0), 700 mM 2-mercaptoethanol, 2% SDS, 0.1% bromophenol blue) and run on discontinuous 7% SDS gels as in [8]. T-Ag (M_r 94 000) was eluted overnight from 2 mm gel slices with 500 μl water containing 10% 2-mercaptoethanol and counted for ^{32}P or ^{35}S.

2.2. *Isoelectric focusing (IEF)*

After dialysis and lyophilization SDS–PAGE purified T-Ag (5–20 μg protein) was dissolved in 50 μl 0.2 M Tris–HCl (pH 7.0), 0.1% SDS, 5 mM dithiothreitol by heating for 1 min at 90°C. In this solution T-Ag was alkylated by incubation with *N*-ethylmaleimide (54 mM) for 1 h as in [9]. The reaction was stopped by the addition of 2-mercaptoethanol (1.42 M). The samples were adjusted to 12% NP40 and 7.6 M deionized urea, incubated for 12 h at 4°C and loaded on the focusing gel near the cathode. Isoelectric focusing was performed in 3.3% polyacrylamide flat gels according to [10] with minor modifications. The gels contained 2% (v/v) carrier ampholytes pH 3–10 (LKB), 8.8 M urea and 2% NP40 and were run at 20 W (2000 V) for 5 h. For autoradiography the dried gels were exposed to Agfa T 4 X-ray film at −70°C. The pH gradients were determined according to [11]. For refocusing experiments in a second dimension, the gel strip containing T-Ag bands was put onto a new flat-bed gel near the cathode. For M_r controls, focusing gels were run in the second dimension without equilibration on SDS–PAGE (10%) according to [11].

3. Results and discussion

SV40 T-Ag isolated by indirect immunoprecipitation and SDS–PAGE was checked routinely for integrity and homogeneity by re-electrophoresis on SDS–PAGE (not shown). Since SDS binds tightly to proteins and influences their charge very strongly, isoelectric focusing of SDS–PAGE purified T-Ag required the removal of SDS by NP40. It had been shown that SDS can be dissociated from proteins such as bovine serum albumin, carbonic anhydrase and poliovirus proteins by forming mixed micelles with NP40 without an influence on the pI [12–15]. According to the DNA sequence data [16,17] T-Ag is rich in cysteine and therefore, the complete denaturation by SDS possibly exposes numerous free SH-groups which have to be blocked by alkylation with *N*-ethylmaleimide (NEM). Without complete reduction and alkylation SDS–PAGE purified T-Ag precipitated almost completely at the starting point of the focusing gels. T-Ag alkylated with NEM entered the focusing gel completely, but it focused in numerous bands between pH 6.5–5 as shown in fig.1A ([^{35}S]methionine-labeled) and C ($^{32}PO_4$-

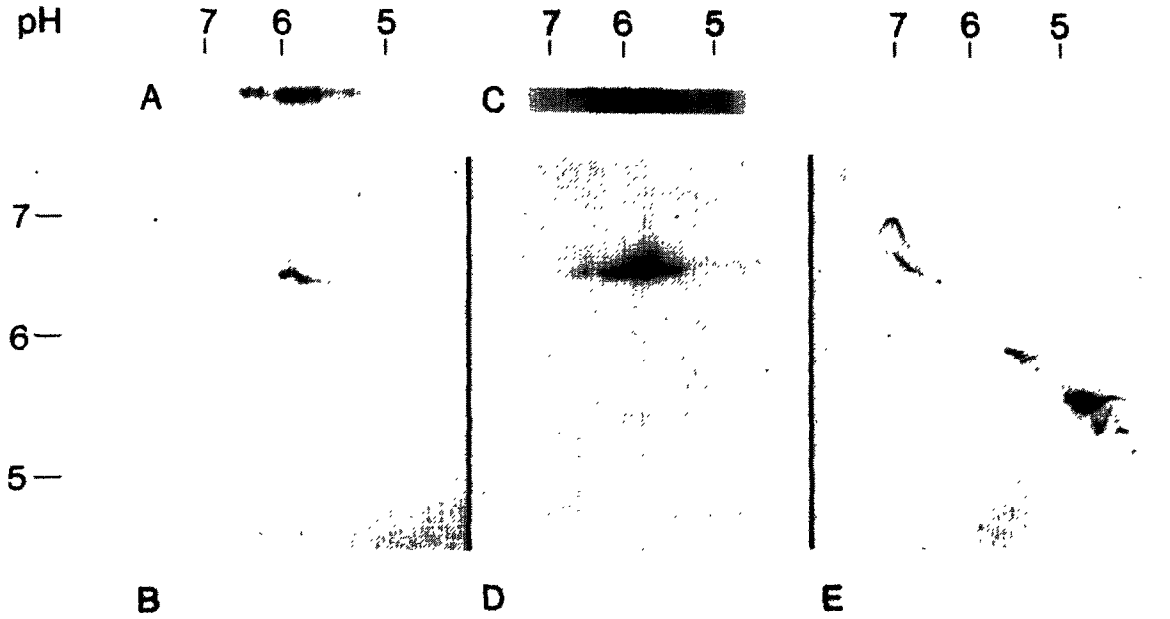

Fig.1. Isoelectric focusing analysis of SV40 T-antigen. T-Ag was isolated from SV80 cells by immunoprecipitation and SDS–PAGE. After elution from the gel slices T-Ag was alkylated with NEM (pH 7, 37°C) as in section 2. T-Ag was loaded onto slab gels containing 8.8 M urea, 2% NP40 and 2% ampholyte, pH 3–10 (LKB) and focused for 5 h at 20 W and 4°C. (A) [^{35}S]Methionine-labeled; (C) $^{32}PO_4$-labeled. For refocusing experiments (B,D,E) the gel strips containing the focused T-Ag bands (A,C) were cut out, put onto a second IEF gel near the cathode and refocused in a second dimension: (B) [^{35}S]methionine; (D) $^{32}PO_4$; (E) gel D stained with Coomassie blue R250. (E) contains the following standard proteins: myoglobin (horse), conalbumin, bovine serum albumin, β-lactoglobulin and ferritin.

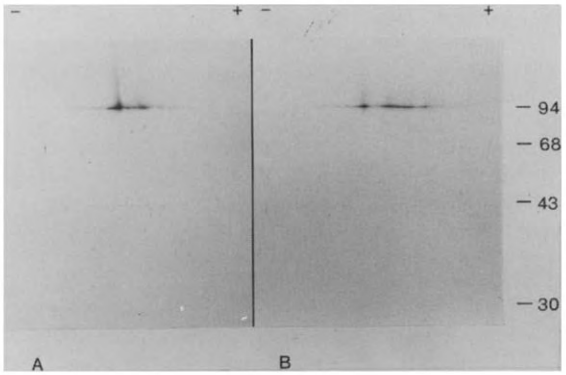

Fig.2. M_r analysis of focused T-Ag bands by a second dimension on SDS–PAGE. T-Ag was isolated and focused as in section 2. The gel strip containing the focused T-Ag bands was cut out, put onto a 10% SDS-gel and SDS–PAGE was performed in a second dimension: (A) [^{35}S]Methionine; (B) [^{32}P]phosphate. M_r markers: phosphorylase A (94 000); bovine serum albumin (68 000); ovalbumin (43 000); carbonic anhydrase (30 000).

labeled). To examine whether all these T-Ag focusing bands represent stable charge isomers, the gel strip containing the T-Ag isoelectric focusing bands was cut out, laid on a second IEF slab gel and refocused in a second dimension. To judge the quality of the second isoelectric focusing dimension, standard proteins with known isoelectric points were added to the first dimension (fig.1C). These proteins refocused in a diagonal as expected for stable charge isomers (visualized in fig.1E by Coomassie blue staining of fig.1D). In contrast, all the acidic T-Ag bands shown in fig.1A,C refocused in a sharp band at pH ~6.5, which sometimes was found to be split in a double band (fig.1B,D). To exclude the possibility that the acidic bands were generated by chemical degradation of T-Ag, T-Ag exhibiting a focusing pattern as shown in fig.1A or C, was examined by SDS–PAGE in a second dimension: re-electrophoresis on SDS–PAGE showed all the focusing bands at the molecular weight of intact T-Ag (M_r 94 000) (fig.2A,B). These results indicate that the formation of the unstable acidic bands of T-Ag is not due to a proteolytic or oxidative degradation of the polypeptide chain.

One likely possibility for the formation of unstable acidic forms of T-Ag may be interactions of T-Ag monomers leading to reversible aggregation patterns. To test this possibility, T-Ag was incubated with bovine serum albumin (BSA) which is known to associate with itself and other proteins [13] under isoelectric focusing conditions. Cofocusing of T-Ag and BSA resulted in a shift of the pH 6.5 band to acid pI values. In addition raising the temperature of the sample to 37°C also produced the acidic banding pattern, which could be shifted reversible into the pH 6.5 band (not shown). T-Ag alkylated at 4°C focused directly at pH 6.5 as a double band as shown by staining with Coomassie blue as well as by autoradiography of [^{35}S]methionine or $^{32}PO_4$ labeled T-Ag (fig.3A–D). Altogether, these data suggest that interactions between T-Ag monomers may be the most likely explanation for the formation of the unstable acidic forms of T-Ag, which can be blocked by lowering the temperature.

Our results show that two differently charged isomers of T-Ag exist. Since T-Ag binds to cellular DNA and RNA, as well as to SV40 DNA [18,19] and this binding depends on differential phosphorylation states of T-Ag [20], further experiments will show whether the two charge isomers are differently phosphorylated and whether these isomers may correlate with different biological activities of T-Ag.

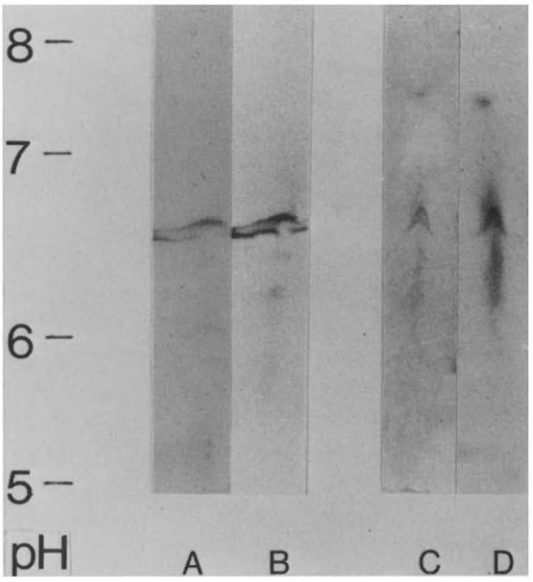

Fig.3. Isoelectric focusing of SDS–PAGE purified T-Ag labeled with either [^{35}S]methionine (A,B) or [^{32}P]phosphate (C,D). Lyophilized T-Ag was dissolved in 0.2 M Tris–HCl (pH 7.0), 0.1% SDS and alkylated with NEM (54 mM, 4 h, 4°C). Focusing was performed as in section 2. (A) [^{35}S]-Methionine labeled T-Ag stained with Coomassie blue R250; (B) autoradiography of gel A; (C) $^{32}PO_4$-labeled T-Ag, stained with Coomassie blue R250; (D) autoradiography of gel C.

Acknowledgements

We are indebted to Dr W. Deppert for his help with this manuscript. The excellent technical assistance of Ms Cornelia Jenkner is warmly acknowledged. This study was supported by the Deutsche Forschungsgemeinschaft (He 882/2, 882/4).

References

[1] Weil, R. (1978) Biochim. Biophys. Acta 516, 301–387.
[2] Tegtmeyer, P., Rundell, K. and Collins, J. K. (1977) J. Virol. 21, 647–657.
[3] Greengard, P. (1978) Science 199, 146–152.
[4] Crawford, L. V. and O'Farrell, P. Z. (1979) J. Virol. 29, 587–596.
[5] Greenspan, D. S. and Carroll, R. B. (1979) 99, 413–416.
[6] Tegtmeyer, P., Schwartz, M., Collins, J. K. and Rundell, K. (1975) J. Virol. 16, 168–178.
[7] Kessler, S. W. (1975) J. Immunol. 115, 1617–1624.
[8] Lämmli, U. K. (1970) Nature 227, 680–685.
[9] Riordan, J. F. and Vallee, B. L. (1972) Methods Enzymol. 25, 449–456.
[10] Görg, A., Postel, W. and Westermeyer, R. (1978) Anal. Biochem. 89, 60–70.
[11] O'Farrell, P. H. (1975) J. Biol. Chem. 250, 4007–4021.
[12] Ames, G. F. and Nikaido, K. (1976) Biochemistry 15, 616–623.
[13] Hamann, A. and Drzenieck, R. (1978) J. Chromatogr. 147, 243–262.
[14] Tuszynski, G. P., Buck, C. A. and Warren, L. (1979) Anal. Biochem. 93, 329–338.
[15] Manrow, R. E. and Dottin, R. P. (1980) Proc. Natl. Acad. Sci. USA 77, 730–734.
[16] Fiers, W. R., Contreras, R., Haegeman, G., Rogiers, R., Van de Voorde, A., Van Heuverswyn, Van Herreweghe, J., Volckaert, G. and Ysebaert, M. (1978) Nature 273, 113–120.
[17] Reddy, V. B., Thimmappaya, B., Dhar, R., Subramanian, K. N., Zain, B. S., Pan, J., Celma, M. L. and Weissman, S. M. (1978) Science 200, 494–502.
[18] Carroll, R. B., Hager, L. P. and Dulbecco, R. (1974) Proc. Natl. Acad. Sci. USA 71, 3754–3757.
[19] Oren, M., Winocour, E. and Prives, C. (1980) Proc. Natl. Acad. Sci. USA 77, 220–224.
[20] Montenarh, M. and Henning, R. (1980) FEBS Lett. 114, 107–110.