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# BIOCHEMICAL AND IMMUNOCHEMICAL CHARACTERIZATION OF TWO SIMIAN VIRUS 40 (SV40)-SPECIFIC GLYCOPROTEINS IN NUCLEAR AND SURFACE MEMBRANES OF SV40-TRANSFORMED CELLS [2]

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SUMMARY

Plasma membranes of several simian virus 40-transformed cells contain virus-specific proteins with molecular masses of ∿ 100,000D and ∿ 60,000D and isoelectric points of ∿ 4.7 and ∿ 4.5, respectively. Triton X-100 extracts of purified nuclei from simian virus 40-transformed hamster lymphocytes contain the same proteins but in different proportions, the high molecular mass component being enriched six-fold in terms of the lower molecular mass one. Both proteins can be labeled metabolically with [$^{14}$C]glucosamine and their isoelectric points altered by neuraminidase treatment, showing that they are sialoglycoproteins.

## INTRODUCTION

The capacity of SV40 to neoplastically transform cells is mediated by two proteins coded by the SV40 A-gene, identified as T-antigen (∿ 100,000D) and t-antigen (∿ 17,000D), which have the same $NH_2$-terminal amino acid sequence (1-6). There is evidence that the T-protein is subject to cellular processing, leading to a variety of derivatives that reside in one or more cellular compartments (7-15) including the nuclei, mitochondria and plasma membranes of SV40-transformed cells.

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[2] Abbreviations used: HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; IE, immune electrophoresis; IEF, isoelectric focusing; IEF-IE, bidimensional isoelectric focusing-immune electrophoresis; IEF-DS-PAGE, bidimensional isoelectric focusing-dodecyl sulfate-polyacrylamide gel electrophoresis; pI, isoelectric point; SV40, simian virus 40; TCA, trichloroacetic acid.

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Robb (10, 13) has examined the nature of SV40 U-antigen which, like T-antigen is nucleus associated but, unlike T-antigen, is thermostable, and has demonstrated that U-antigenicity represents site(s) near the COOH-terminus of the T-antigen. Further, Deppert (14) has presented data interpreted to show that U-antigen, unlike T-antigen, has a preferential association with a nuclear matrix prepared by extracting nuclei of SV40-transformed cells with a non-ionic detergent, treating with DNase and further extracting with 1.5M NaCl.

We have applied biochemical and immunochemical techniques to characterize the membrane proteins characteristic of certain SV40-transformed cells (9, 12, 13), in particular GD248 lymphocytes (16). We have discovered two classes of SV40-specific plasma membrane proteins, pI $\sim$ 4.5/$\sim$ 60,000D and pI $\sim$ 4.7/$\sim$ 100,000D. Both proteins also occur on intracellular membranes, albeit in proportions different from those in plasma membranes (12). We now show that these SV40-specific proteins are glycoproteins and that the $\sim$ 100,000D/pI $\sim$ 4.7 component is enriched in the nuclear envelope *vs.* other membrane compartments (17).

## MATERIALS AND METHODS

All procedures and materials not specified here were as described before (12, 13, 18). Neuraminidase (Lot 1275A, Behring Diagnostics) was obtained from American Hoechst Corp. (Somerville, N. J.), glucosamine hydrochloride (D-[1-$^{14}$C]; specific activity 5-10 mCi/mmole) from New England Nuclear (Boston, MA.), RPMI 1640 medium from Associated Biomedic Systems, Inc. (Buffalo, N. Y.) heat-inactivated, fetal calf serum from GIBCO (Grand Island, N. Y.), and fluorescein-conjugated immunoglobulin, (IgG)-fraction from rabbit against guinea pig IgG and hamster IgG from Cappel Laboratories, Inc. (Cochranville, Penn.). SV40-transformed hamster GD248 lymphocytes were propagated as solid lymphomas and lymphocyte membranes prepared therefrom as described in refs. 19 and 20. Nuclei were isolated as in (21), except that GD248 cells were suspended in 65 mM NaCl/75 mM KCl/5 mM $MgCl_2$/10 mM, HEPES, pH 7.0 before disruption by nitrogen decompression (450 psi for 15 min). Nuclei were pelleted at 1.5 $\cdot$ $10^4$g $\cdot$ min, washed once, layered ($10^8$ nuclei/ml disruption buffer) onto 7 ml of 1.8M sucrose, 70 mM KCl/2.5 mM $MgCl_2$/20 mM HEPES, pH 7.0. After centrifugation at 6.5 $\cdot$ $10^5$g $\cdot$ min (Beckman SW41 rotor), the nuclei were washed twice in disruption buffer. Recovery of nuclei was monitored by counting of Trypan blue-stained preparations. Contamination of the nuclear preparation was monitored using marker enzymes for plasma membranes, endoplasmic reticulum, lysosomes and mitochondria (19). For metabolic labeling about 5 $\cdot$ $10^8$ ($10^7$/ml in RPMI 1640 medium) cells were labeled with [$^{14}$C]glucosamine (2 µCi/ml of medium) for 6-12 hrs, washed twice in Dulbecco's phosphate-buffered saline, pH 7.2, and subcellular fractionation was carried out as in (19). Isotope incorporated into glycoproteins of whole cells and subcellular fractions was determined after precipitation and two washes with ice-cold 10% TCA.

For IEF, IEF-IE and IEF-DS-PAGE, plasma membrane proteins were solubilized as before (12, 13, 18). Nuclear envelope proteins were extracted from 5 $\cdot$ $10^8$ nuclei/

ml, using 65 mM NaCl/ 75 mM KCl/5 mM $MgCl_2$/10 mM HEPES, pH 7.0, 0.5% Triton X-100. After 5-fold dilution with cold buffer, the extracted nuclei were collected at $1.5 \cdot 10^4$ g · min, the supernatant fluid, cleared at $10^7$ g · min, dialyzed for 16 h against 1 mM HEPES, pH 8.5, 0.2% Triton X-100, and adjusted to a protein concentration of ∿ 5 mg/ml by partial lyophilization.

Immunochemical analyses employed guinea pig sera raised against GD248 membranes (12, 13). SV40 T-antigen was monitored using sera of tumor-bearing animals yielding a positive intranuclear indirect immune fluorescence with SV40 transformed cells or isolated nuclei thereof (9). Nuclei were analyzed before and after treatment with Triton X-100.

For quantitation of metabolic incorporation of [$^{14}$C]glucosamine into membrane glycoproteins, fixed and Coomassie blue stained gels were scanned photometrically and cut into 1 mm slices, allowing us to express the relative distribution of $^{14}$C-activity in terms of Coomassie blue staining.

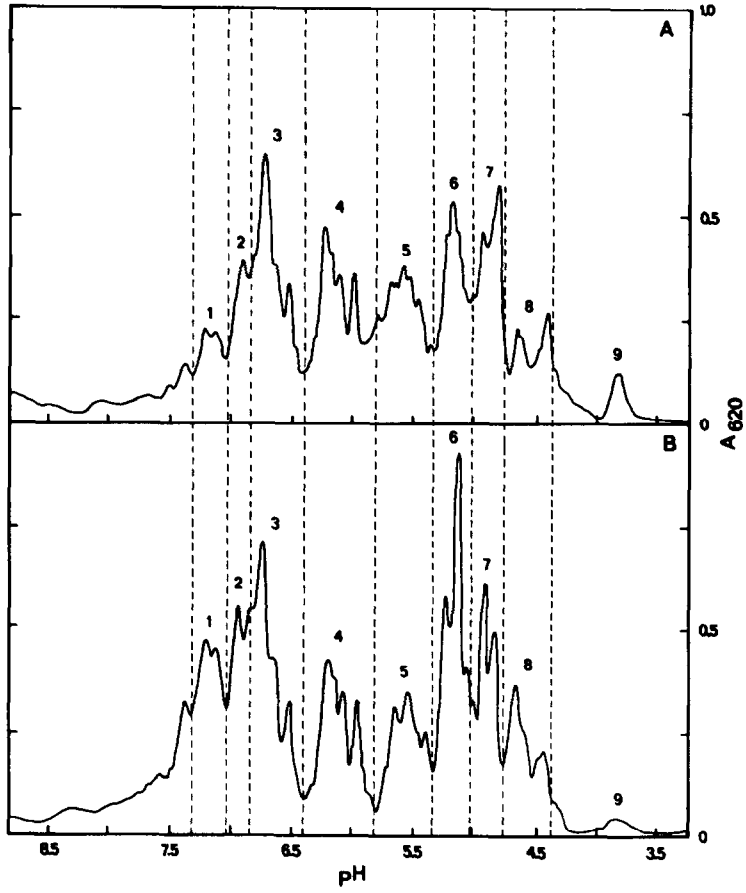



Figure 1: IEF of Triton solubilized plasma membranes (A) and nuclear envelopes (B) from GD248 cells (0.1 mg membrane protein each). The abscissa gives the pH gradient and the ordinate the absorbance at 620 nm for Coomassie blue protein staining.

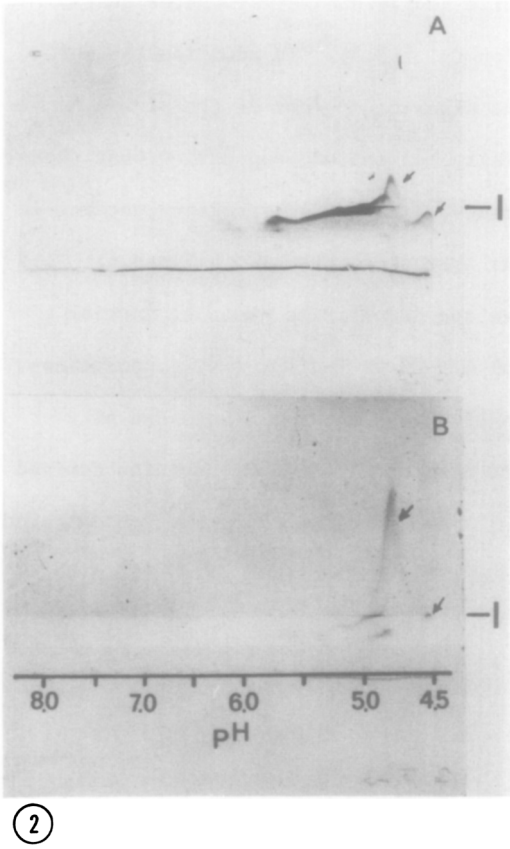
RESULTS

Purification of nuclei from GD248 cells (21) yielded nuclei with intact envelopes and free of other subcellular organelles. By centrifugation through 1.8 M sucrose, we recovered 75-80% of the nuclei and less than 4% of marker enzymes characteristic for other subcellular organelles. Immune fluorescence (9) of purified nuclei showed a strong, thermostable fluorescence at the nuclear periphery but no intranuclear fluorescence, when reacted with anti GD248-membrane serum followed by fluorescein-conjugated rabbit anti-hamster IgG. This immunofluorescence, unlike the T-antigen reaction, was substantially reduced by extraction with 0.5% Triton X-100 at 37°C for 5-10 min.

IEF of Triton X-100-solubilized proteins of plasma membranes, or nuclei, yields a complex protein pattern which can be divided into eight major zones between pH 8.0 and 4.5 (18; Figure 1 A,B). Some quantitative differences and several qualitative differences are apparent in zones A, 6 and 7, and two strong bands at pI $\sim$ 5.2 and pI $\sim$ 4.9. The pI $\sim$ 4.5 component is equally prominent in both plasma membranes and nuclear extracts but the pI $\sim$ 4.7 component is much more conspicuous in the latter.

Bidimensional IEF-IE (Fig. 2) shows that the complex precipitates between pI 6.5 and 4.8, found with GD248 plasma membranes (13) are almost completely absent in the nuclear extracts (suggesting elimination of many plasma membrane antigens). However, the SV40-specific pI $\sim$ 4.7 and pI $\sim$ 4.5 antigens are clearly present in the nuclear extract. The height of the pI $\sim$ 4.7 precipitation arc measured from the interface, I, between antiserum-free and antiserum-containing agarose is 3-times that of plasma membranes (identical amounts of protein). The results suggest preferential localization of the pI $\sim$ 4.7 component in the nuclear envelope. The height of the pI $\sim$ 4.5 component, in contrast, suggests equivalent concentrations in plasma membrane and nuclear extracts (Fig. 2B).

[$^{14}$C]glucosamine-labeling of GD248 cells for 16 h yielded incorporation of 3% of the total label added, i.e. $8.3 \cdot 10^5$ cpm of $2.5 \cdot 10^7$ cpm, 10-15% of which was found in the plasma membrane fraction. As shown in Fig. 3A, B, most of [$^{14}$C]-



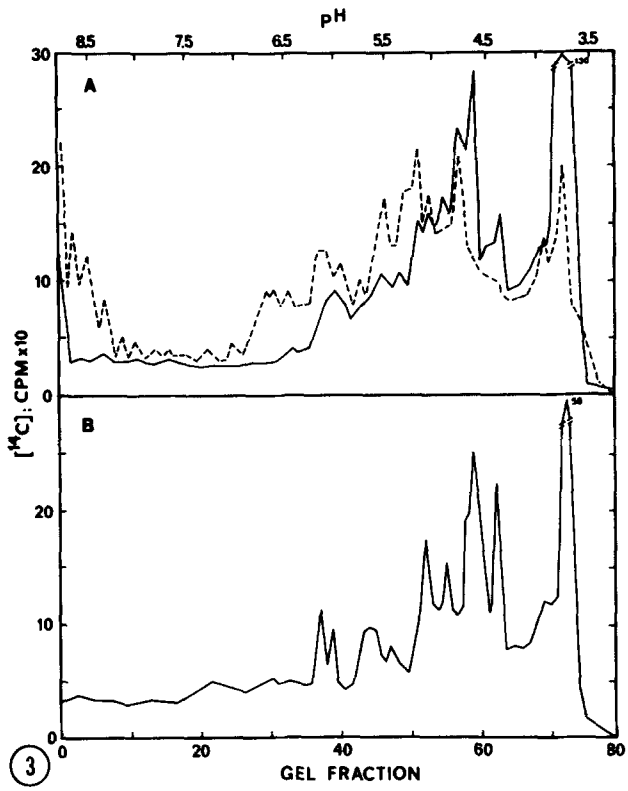




Figure 2: Bidimensional IEF-IE of plasma membranes (A) and nuclear envelopes (B) First dimension (horizontal): solubilized membranes (0.4 mg protein) focused in polyacrylamide gel slabs. Second dimension (vertical): electrophoresis into antiserum-containing agarose (46 µl anti-GD248-membrane serum/ml agarose). The pH gradient has been superimposed at the position of the focusing gel (immune plate B).

Figure 3: IEF of plasma membranes (A) and nuclear envelopes (B) from GD248 cells metabolically labeled with [$^{14}$C]glucosamine. For each preparation 0.1 mg of membrane protein was focused in cylindrical gels. The abscissa gives the pH-gradient and the ordinate the $^{14}$C-activity. Neuraminidase treatment (50 U/ml) of isolated GD248 plasma membranes (2 mg/ml) metabolically labeled with [$^{14}$C]glucosamine in 10 mM HEPES, 0.2 mM $MgCl_2$, 0.2 mM $CaCl_2$, pH 6.0, at 37°C for 1 hr. After adjusting the pH to 7.4, the membranes were washed and processed for protein analysis as in (18).
Solid line: Control pattern. Broken line: After neuraminidase treatment of isolated plasma membranes.

glucosamine was incorporated into periodate-Schiff-positive components (18) of the membrane and the nuclear envelope. Importantly, the SV40-specific proteins, at pI ∿ 4.7 and pI ∿ 4.5, were strongly labeled, regardless of origin, indicating that these are glycoproteins.

Treatment of the GD248 plasma membranes with neuraminidase eliminates nearly all the radioactivity in the ganglioside region (pI ∿ 3.5). In addition the more acid portion of the pI ∿ 4.7 component and the alkaline segment of the pI ∿ 4.5 band (Fig. 3A; cf. also Fig. 1) were almost fully eliminated, suggesting that these entities are sialylated, at least in part. However, both SV40-specific proteins in the nuclear envelope remained heavily labeled. Moreover, the pI ∿ 4.7 and pI ∿ 4.5 components of plasma membrane and nuclear envelope demonstrate at least partial antigenic identity (Fig. 2A,B). Bidimensional IEF-DS-PAGE after [$^{14}$C]glucosamine-labeling (Fig. 4) documents the incorporation of [$^{14}$C]glucosamine into the pI ∿ 4.5 and pI ∿ 4.7 IEF components and the ∿ 60,000D and ∿ 100,000D proteins derived therefrom upon IEF-DS-PAGE. In case of the pI ∿ 4.5 band, labeling was concentrated in the more alkaline of the overlapping DS-PAGE derived therefrom (Fig. 4).

## DISCUSSION

We previously demonstrated ∿ 100,000D/pI ∿ 4.7 and ∿ 60,000D/pI ∿ 4.5 proteins in plasma membranes purified from several types of SV40-transformed cells, including GD248 lymphocytes, lacking in membranes of normal cells or cells transformed by polyoma virus (12, 13). Our present studies show that Triton X-100 extraction of GD248 nuclei suppresses the SV40-specific immunofluorescence at the nuclear periphery and elutes the ∿ 100,000D/pI ∿ 4.7 and ∿ 60,000D/pI ∿ 4.5 SV40-specific proteins which, by all criteria applied, are similar to the SV40-induced plasma membrane proteins described before (12, 13).

Labeling with [$^{14}$C]glucosamine, under conditions avoiding label reutilization indicates that the ∿ 100,000D/pI ∿ 4.7 protein is a glycoprotein and its susceptibility to the action of neuraminidase suggests that it is a sialoglycoprotein. The SV40-specific, ∿ 60,000D/pI ∿ 4.5 protein is also glycosylated. Both entities reveal some microheterogeneity, upon bidimensional IEF-DS-PAGE, particularly after neuraminidase treatment. This is apparent as small differences in molecular masses and non-homogeneous immunoprecipitates and may reflect non-uniform glycosylation of a single category of polypeptide chain.

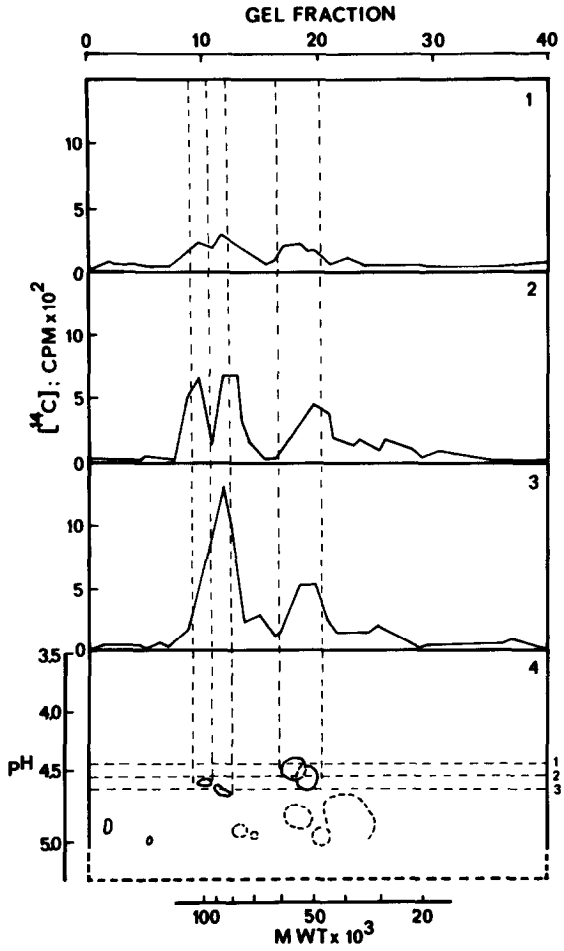



Figure 4: Bidimensional IEF-DS-PAGE of plasma membranes metabolically labeled with [$^{14}$C]glucosamine. First dimension: solubilized membrane (0.4 mg protein) was focused in cylindrical gels. Second dimension: after equilibration with 3% DS and 120 mM DTT the focused proteins were electrophoresed into an acrylamide gel slab (partly shown in panel 4). To quantitate the $^{14}$C-activity of the DS-PAGE components derived from the pI $\sim$ 4.5 and $\sim$ 4.7 focusing bands, Coomassie blue stained gel slabs from IEF-DS-PAGE, were cut into strips corresponding to the pH 4.5 (panel 1), 4.6 (panel 2) and 4.7 (panel 3) regions of the focusing gel. Each of these strips was sliced into 40 2 mm fractions and counted after dissolution in 0.6 ml 20% perchloric acid. The abscissas give molecular masses (panel 4) and gel fractions (panel 1-3) and the ordinates the pH gradient (panel 4) and the $^{14}$C-activity (panels 1-3). Numbers 1-3 (panel 4) indicate centers of slices represented in panels 1-3.

Our results indicate that (a) glycosylation can be one step in the host-cell processing of SV40-induced proteins and (b) very similar, host-cell processed,

SV40-derived proteins can occur in more than one cellular membrane compartment, with the ∿ 100,000D/pI ∿ 4.7 protein preferentially concentrated at/near the nuclear envelope.

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