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Acylated simian virus 40 large T-antigen: a new subclass associated with a detergent-resistant lamina of the plasma membrane

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We have analyzed the plasma membrane association of the SV40 large tumor antigen (large T) in SV40-transformed BALB/c mouse tumor cells (mKSA). Isolated plasma membranes were subfractionated: treatment with the non-ionic detergent Nonidet P40 (NP40) resulted in a NP40-resistant plasma membrane lamina, which could be further extracted with the zwitterionic detergent Empigen BB. Analysis of the different plasma membrane fractions revealed that only about one third of large T associated with isolated plasma membranes could be solubilized with NP40. The residual plasma membrane-associated large T was tightly bound to the NP40-resistant lamina of the plasma membrane from which it was released by treatment with the zwitterionic detergent Empigen BB. Further evidence for a specific interaction of a distinct subclass of large T with the plasma membrane was provided by showing that only T associated with the NP40-resistant lamina of the plasma membrane contained covalently bound fatty acid. Neither nuclear large T nor large T in the NP40-soluble plasma membrane fraction could be labeled with [^{3}H]palmitic acid. Our results indicate that an acylated subclass of large T interacts specifically with a structure of the plasma membrane, suggesting that it might be involved in a membrane-dependent biological function.

Key words: fatty acid acylation/plasma membrane transforming proteins/plasma membrane lamina/SV40 large T-antigen

Introduction

To bring about the pleiotropic changes which are involved in transformation, the transforming proteins of tumor viruses must interact with different structural systems in the cell. As a consequence, these proteins are found in different subcellular locations. In view of the crucial role postulated for the plasma membrane in the regulation of cell morphology and cell growth (Edelman, 1976), the finding that several transforming proteins are, at least in part, plasma membrane-associated (Courtneidge *et al.*, 1980; Krueger *et al.*, 1980, 1982; Krzyek *et al.*, 1980; Willingham *et al.*, 1979, 1980; Ito *et al.*, 1977; Ito, 1979; Carmichael *et al.*, 1982; Witte *et al.*, 1979), has gained considerable attention: it has been suggested that these proteins might specifically interact with membrane or cytoskeletal components and thereby affect cellular morphology and adhesion (Courtneidge *et al.*, 1980; Krueger *et al.*, 1980, 1982; Krzyek *et al.*, 1980; Willingham *et al.*, 1979, 1980; Ito *et al.*, 1977; Ito, 1979; Carmichael *et al.*, 1982; Witte *et al.*, 1979; Hynes, 1980; Rohrschneider, 1980; Boschek *et al.*, 1981).

Given the problems associated with cell fractionation, the determination of the subcellular location of viral transforming proteins poses problems. Nevertheless, evidence for a specific plasma membrane association could be provided for those transforming proteins located primarily at the plasma membrane, for example pp60 src, the transforming protein of Rous sarcoma virus (RSV) (Courtneidge *et al.*, 1980; Krueger *et al.*, 1980, 1982; Krzyek *et al.*, 1980; Willingham *et al.*, 1979), or for the medium T-antigen of polyoma virus (Py) (Ito *et al.*, 1977; Ito, 1979; Carmichael *et al.*, 1982). In contrast, the major transforming protein of SV40, the SV40 large tumor antigen (large T) (reviewed in Tooze, 1981) is located mainly in the cell nucleus (Pope and Rowe, 1964; Soule and Butel, 1979), although small amounts of this molecule are detected also in plasma membrane fractions (Soule and Butel, 1979) and on the cell surface (Deppert *et al.*, 1980; Soule *et al.*, 1980; Henning *et al.*, 1981). However, since extracted large T can bind to plasma membranes during cell fractionation (Deppert and Walter, 1976) and adsorb to the surface of living cells (Lange-Mutschler and Henning, 1982) the origin of plasma membrane-associated large T has not been clear. The association of large T with the plasma membrane might be an artefact of the preparation procedures. On the other hand, a plasma membrane-associated subclass of large T might be necessary for viral replication or viral transformation. A final proof that large T is genuinely associated with the plasma membrane will depend on showing that: (i) large T interacts specifically with the plasma membrane; and (ii) that plasma membrane-associated large T can be distinguished biochemically, e.g., by means of a different post-translational modification, from nuclear large T. To investigate these questions, we have analyzed whether large T is associated with a detergent-resistant fraction of isolated plasma membranes. These studies were initiated by the finding that, by cell fractionation, the majority of pp60 src, as well as of Py medium T-antigen was found in the plasma membrane fraction (Courtneidge *et al.*, 1980; Krueger *et al.*, 1980, 1982; Krzyek *et al.*, 1980; Willingham *et al.*, 1979; Ito *et al.*, 1977; Ito, 1979; Carmichael *et al.*, 1982). On the other hand, mild detergent treatment of cells *in situ* released only a fraction of these proteins, and considerable amounts remained associated with the cytoskeleton (Burr *et al.*, 1980; Schaffhausen *et al.*, 1982), furthering the idea that these proteins may act on plasma membrane-associated cytoskeletal elements. In addition, we have asked, whether nuclear and plasma membrane-associated large T can be differentiated by a post-translational modification specific for a variety of plasma membrane-associated proteins, including pp60 src (Sefton *et al.*, 1982), namely fatty acid acylation (Sefton *et al.*, 1982; Schlesinger, 1981; Schmidt, 1982; Schlesinger *et al.*, 1980; Omary and Trowbridge, 1981).

Results

Cell fractionation and subfractionation of plasma membranes

The plasma membrane can be subfractionated according to the different solubilities of its constituents: treatment with non-ionic detergents yields a substructure, which still contains

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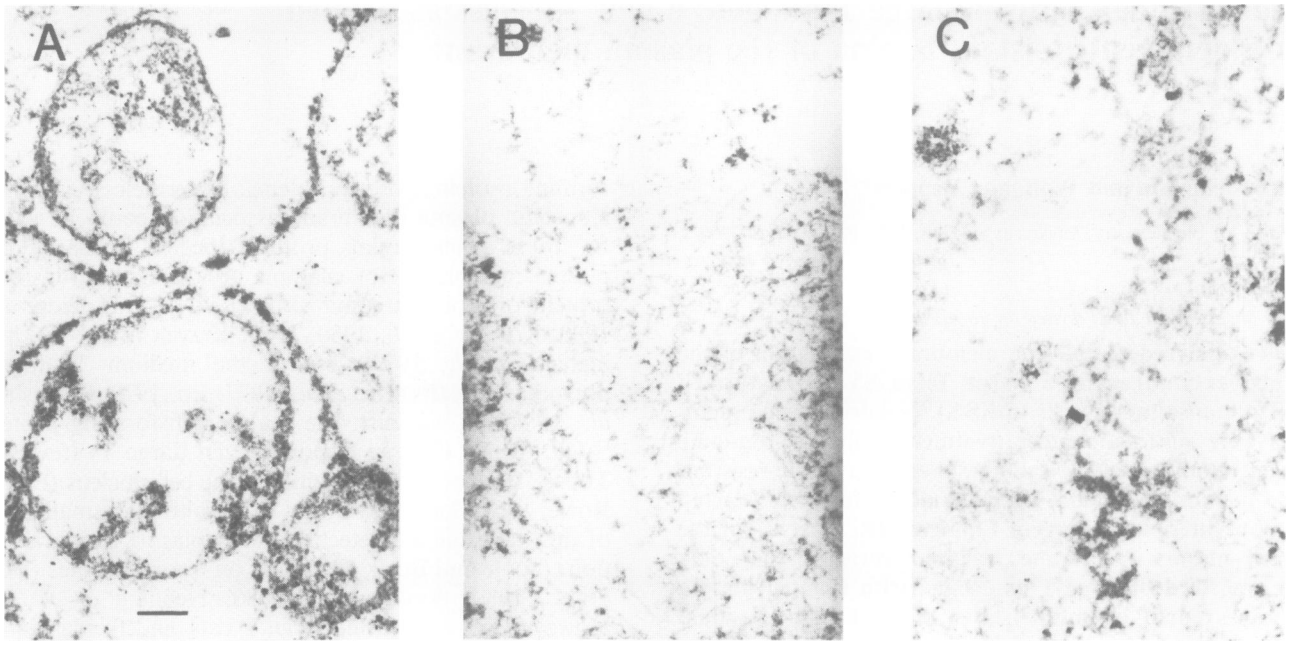

Fig. 1. Electron micrographs of plasma membranes and of plasma membrane subfractions isolated from mKSA cells. Thin sections of plasma membranes **(A)**, the NP40-resistant plasma membrane fraction **(B)** and the residual plasma membrane fraction after Empigen BB treatment **(C)** were processed for electron microscopy as described in Materials and methods. Bar represents 0.1 μm.

many membrane and cell surface proteins as well as some specifically retained membrane lipids (Mescher *et al.*, 1981). Subfractionation can be performed *in situ*. In this case, the detergent-resistant fraction of the plasma membrane is still connected with the cytoskeleton, suggesting that it forms a submembraneous lamina directly underlying the plasma membrane into which membrane and cell surface proteins are anchored (Ben-Ze'ev *et al.*, 1979; Carter and Hakomori, 1981). This structure is also obtained after detergent treatment of isolated plasma membranes (Mescher *et al.*, 1981).

Fractionation *in situ* is not feasible with SV40-transformed cells, since even mild detergent treatment releases the majority of nuclear large T (Staufenbiel and Deppert, 1983), and thereby may lead to a secondary adsorption of nuclear large T to cytoskeletal and plasma membrane components. Therefore, we have used a cell fractionation procedure based on the method of Brunette and Till (1971) with several modifications, aimed at minimizing possible artefacts during subfractionation of the plasma membrane (see Materials and methods). Plasma membranes were obtained in high yield (~2% of the total cellular protein), and had the same morphological appearance in phase contrast microscopy as shown earlier (Deppert *et al.*, 1977). They were largely free of nuclear contaminants as judged by phase contrast microscopy.

Isolated plasma membranes were first extracted with NP40 (see Materials and methods) and their NP40-resistant component was further treated with the zwitterionic detergent Empigen BB. This detergent was chosen because we recently found that Empigen BB releases large T from the nuclear matrix (Staufenbiel and Deppert, 1983), a nuclear substructure also insoluble in non-ionic detergents (Berezney and Coffey, 1977). Figure 1 shows electron micrographs of thin sections of isolated mKSA plasma membranes (Figure 1A), and of the NP40-resistant plasma membrane component before (Figure 1B), and after (Figure 1C) treatment with Empigen BB. Plasma membrane vesicles (Figure 1A), in addition to other cytoplasmic material, contain substantial amounts of intracellular membranes, including rough endoplasmic reticulum. After treatment of isolated plasma membranes with NP40 (Figure 1B), a unit membrane structure is no longer visible. Most of the trapped cytoplasmic material has been released and intracellular membranes are dissolved by the detergent treatment. The NP40-resistant plasma membrane component still exhibits some structural organization, with a sometimes fibrillar or filamentous appearance. After Empigen BB treatment (Figure 1C) the structural features of the NP40-resistant plasma membrane component are further reduced and distinct structural elements can no longer be discerned.

The polypeptide pattern of extracts and subcellular structures obtained during fractionation of mKSA cells is shown in Figure 2. H1 and the core histones served as markers for contamination of the cytoplasmic fraction and the plasma membrane fractions by nuclei. Figure 2b (cytoplasmic fraction) and Figure 2d–h (plasma membrane fractions) show that neither histone H1 nor core histones were present in these fractions in significant amounts, supporting the phase contrast microscopic observation that these fractions were largely free of nuclei.

The different plasma membrane subfractions (Figure 2e–h) are characterized by distinct polypeptide patterns. This shows that the subfractionation of the isolated plasma membranes was indeed based on different solubility properties of its individual constituents and did not arise from incomplete extraction. As described by Mescher *et al.* (1981), actin represented the major protein of the NP40-insoluble plasma membrane component (Figure 1e). By treatment with Empigen BB, the plasma membrane-associated actin was almost quantitatively solubilized (Figure 1h). Vimentin in the NP40-resistant plasma membrane component (Figure 1e) was also removed by Empigen BB treatment (Figure 1h).

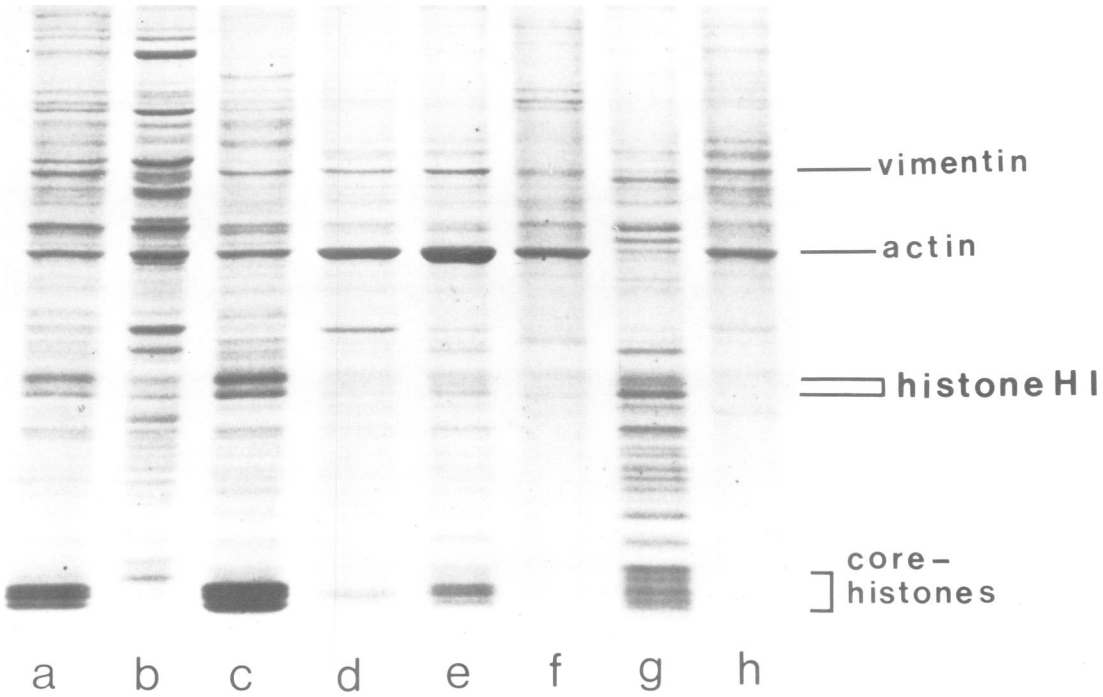

Fig. 2. Polypeptide pattern of different subcellular fractions obtained during cell fractionation of mKSA cells; Coomassie stained SDS-polyacrylamide gel. Cell fractionation was performed as described in the text and in Materials and methods. Approximately 15–20 μg of each fraction was applied to a 7.5–20% hyperbolic gradient gel and run for 6 h at 12 mA. After electrophoresis the gel was stained with Coomassie Brilliant Blue R250. The sample order is: **(a)** homogenate, **(b)** cytoplasmic fraction, **(c)** nuclear fraction, **(d)** total plasma membrane fraction, **(e)** NP40-insoluble plasma membrane fraction, **(f)** NP40-soluble plasma membrane fraction, **(g)** residual plasma membrane fraction after Empigen BB treatment, **(h)** extract of the Empigen BB-treated NP40-insoluble plasma membrane fraction.

Association of large T with the NP40-resistant plasma membrane component

To analyze whether large T might be specifically associated with any subfraction of the plasma membrane, we have determined the relative amount of this molecule in the different subcellular fractions. mKSA cells were pulse-labeled with [^{35}S]methionine for 4 h and subfractionated as described above. The cytoplasmic fraction, an extract of the nuclear fraction prepared according to Schwyzer (1977), the NP40-soluble plasma membrane fraction and the Empigen BB extract of the NP40-insoluble plasma membrane component were immunoprecipitated with anti-SDS-T serum (Deppert and Pates, 1979) and analyzed by SDS-polyacrylamide gel electrophoresis (see Materials and methods).

Figure 3A shows that large T can be detected in all subcellular fractions. As expected, the vast majority of large T was recovered from the nuclear fraction (N). Only a very small amount was present in the cytoplasmic fraction (C), indicating the good structural preservation of the nuclei during cell fractionation. We have not investigated the origin of this cytoplasmic large T. Treatment of isolated plasma membranes with NP40 under our conditions solubilized approximately half of the plasma membrane proteins. Since NP40 treatment had solubilized, in addition to a variety of genuinely plasma membrane-associated proteins (Mescher *et al.*, 1981; Ben-Ze'ev *et al.*, 1979), cytoplasmic material included in plasma membrane vesicles (see Figure 1A) and non-membrane proteins adsorbed to the plasma membrane during cell fractionation, this NP40-soluble plasma membrane fraction comprised proteins of different origin. Immunoprecipitation of this fraction (Figure 3A, NP40 PM) yielded approximately one third of the total plasma membrane-associated large T. However, due to the heterogeneous composition of the NP40 plasma membrane extract, one cannot decide whether large T found in this fraction is specifically plasma membrane-associated, or had adsorbed to the plasma membrane during preparation. In contrast, one can assume that large T solubilized from the NP40-insoluble plasma membrane component by Empigen BB treatment (Figure 3A, Emp PM) represents a subclass specifically interacting with this plasma membrane substructure: large T was not released from this structure by all other treatments known to solubilize the majority of large T from the nucleus, including large T extraction buffers (Griffin *et al.*, 1978; Crawford and O'Farrell, 1979; Tegtmeyer *et al.*, 1975; Ahmad-Zadeh *et al.*, 1976) and high salt (up to 2 M NaCl). Also, treatment with buffers containing various other non-denaturing detergents (Triton X-100, Triton X-114, deoxycholate) did not solubilize significant amounts of large T from the NP40-resistant plasma membrane component. A small portion of large T (~ 10–20% of the amount solubilized with Empigen BB), however, was

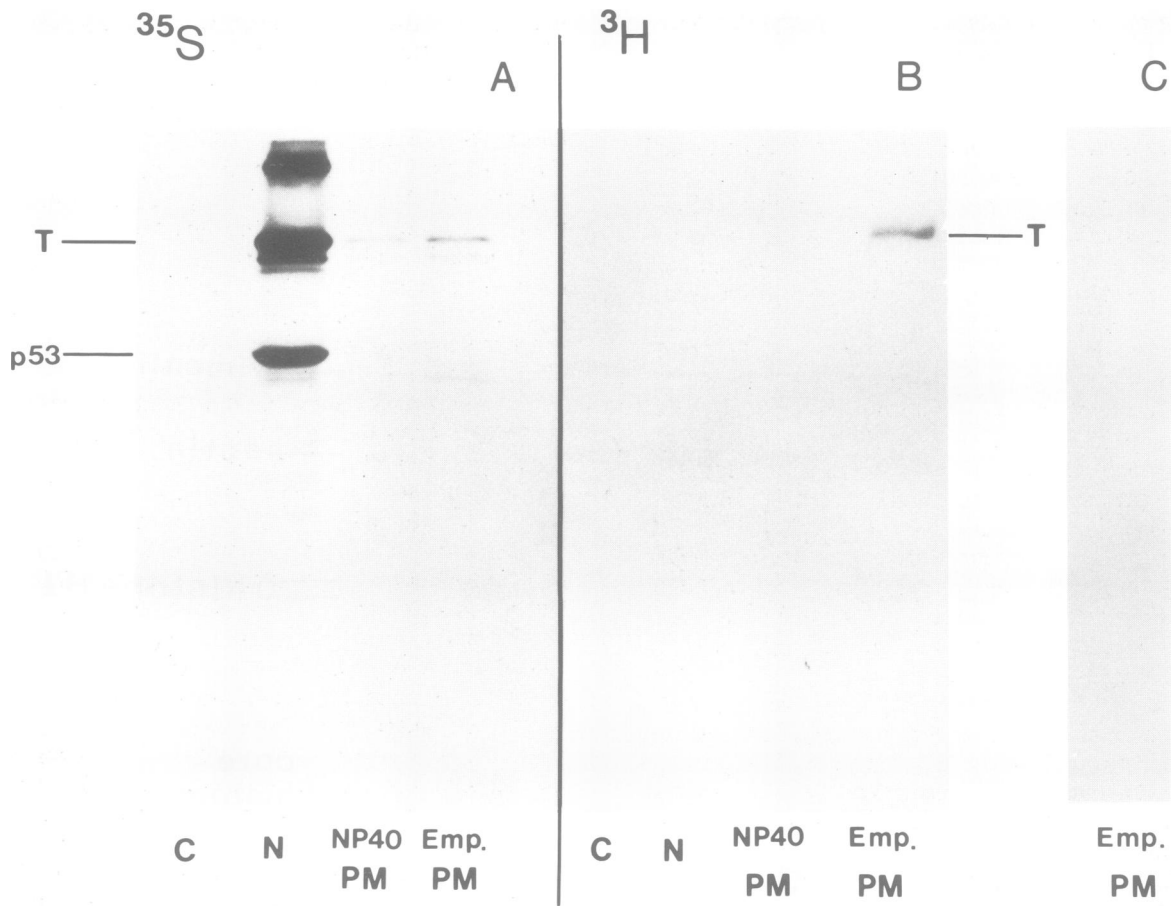

Fig. 3. [^{35}S]Methionine and [^{3}H]palmitic acid-labeled large T in different subcellular fractions of SV40-transformed mKSA cells; SDS-polyacrylamide gel fluorograms. Parallel cultures of [^{35}S]methionine **(A)** and [^{3}H]palmitic acid **(B)** labeled mKSA cells were subfractionated. Extracts were prepared and immunoprecipitated as described in Materials and methods. Aliquots of the immunoprecipitates were applied to a 11.5% acrylamide slab gel and run for 6 h at 12 mA. Large T was immunoprecipitated from the cytoplasmic fraction (C), the nuclear fraction (N), the NP40-soluble plasma membrane fraction (NP40 PM) and the extract of the Empigen BB-treated NP40-insoluble plasma membrane fraction (Emp PM). The ^{3}H label (in **B**, EMP PM) was removed after treatment of the gel with 1 M hydroxylamine for 1 h **(C)**.

released by extraction with RIPA buffer (Gilead *et al.*, 1976), containing a combination of several ionic and non-ionic detergents, including SDS (Klockmann and Deppert, 1983a).

Large T associated with the NP40-resistant plasma membrane component is acylated

Our results so far have shown that two forms of large T can be isolated from plasma membranes: a loosely associated fraction, soluble in NP40, and a fraction tightly associated with an NP40-resistant plasma membrane component. Is it possible to discriminate between these two plasma membrane-associated forms on the basis of a different post-translational modification? This question was prompted by our recent observation that plasma membrane-associated carboxy-terminal fragments of large T, encoded by the Ad2^{+}SV40 hybrid virus Ad2^{+}ND2, as well as plasma membrane-associated large T in mKSA cells, are specifically acylated, whereas their nuclear and cytoplasmic forms are not (Klockmann and Deppert, 1983a, 1983b). In an experiment performed in parallel with the one described for the detection of large T in subfractions of [^{35}S]methionine-labeled cells (see above), the corresponding fractions of mKSA cells after labeling with [^{3}H]palmitic acid (see Materials and methods) were analyzed for the presence of ^{3}H-labeled large T. The result is shown in Figure 3B. No ^{3}H-labeled nuclear large T could be detected (Figure 3B,N) although, after labeling with [^{35}S]methionine, nuclear large T constituted the vast majority of the large T of the cells (Figure 3A,N). ^{3}H-Labeling of large T also could not be detected in the cytoplasmic fraction (Figure 3B,C) or in the NP40-soluble plasma membrane fraction (Figure 3B, NP40 PM). Only large T associated with the NP40-insoluble plasma membrane component was specifically labeled with [^{3}H]palmitic acid and was immunoprecipitated from the Empigen BB extract of this structure (Figure 3B, Emp PM).

Several criteria indicate that the ^{3}H label associated with large T in the NP40-insoluble plasma membrane fraction represents covalently attached fatty acid: (i) the ^{3}H label was still bound to large T after extraction of the plasma membrane with a non-ionic (NP40) and with a zwitterionic (Empigen BB) detergent; (ii) the fatty acid could not be removed from large T by boiling the sample in 3% SDS for 5 min; (iii) the fatty acid was still bound to large T after SDS-polyacrylamide gel electrophoresis; (iv) most importantly, we can exclude that the ^{3}H label associated with large T results from conversion of [^{3}H]palmitic acid into [^{3}H]amino acids which then might have been incorporated into the polypeptide backbone of this molecule: the ^{3}H label could be quantitately removed from large T, when the gel shown in Figure 3B was treated with hydroxylamine (Figure 3C), a treatment shown

to result in a nucleophilic cleavage of many acyl derivatives (Omary and Trowbridge, 1981).

Discussion

Despite accumulating evidence for the presence of large T on the surface of SV40-transformed cells, the origin and the specificity of the plasma membrane location of this molecule had not been clarified with certainty. Lange-Mutschler and Henning (1982) reported the stable binding of extracted large T to the surface of transformed as well as non-transformed cells. They favored the interpretation that large T is a peripheral rather than an integral membrane protein. On the other hand, using an immunological approach, Deppert and Walter (1982) recently showed that only certain domains of large T are exposed on the surface of formaldehyde-fixed SV40 transformed cells, arguing for a specific orientation of large T in the plasma membrane. Here we provide structural and biochemical evidence that a subclass of large T interacts specifically with the plasma membrane. (i) A major fraction of large T found in isolated plasma membranes is tightly associated with a detergent-resistant plasma membrane component. This fraction of large T cannot be solubilized by treatments usually used to extract large T (Griffin *et al.*, 1978; Crawford and O'Farrell, 1979; Tegtmeyer *et al.*, 1975; Ahmad-Zadeh *et al.*, 1976), except by treatment with the zwitterionic detergent Empigen BB. It is, therefore, possible that, due to its unusual solubility properties, this large T subclass had been missed in previous investigations. (ii) Of the many subclasses of large T (Carrol and Gurney, 1982; Gurney *et al.*, 1980; Deppert, 1979; Deppert *et al.*, 1980; Fanning *et al.*, 1981, 1982; Gidoni *et al.*, 1982; Bradley *et al.*, 1982; McCormick and Harlow, 1980; Greenspan and Carroll, 1981; Harlow *et al.*, 1981; Osborn and Weber, 1975) only this one is fatty acid acylated, a post-translational modification specific for a variety of plasma membrane proteins (Schlesinger, 1981; Schmidt, 1982; Schlesinger *et al.*, 1980; Omary and Trowbridge, 1981).

In addition to acylated large T in the detergent-resistant plasma membrane component, we also found non-acylated large T in the NP40-soluble plasma membrane fraction. It is conceivable that the acylated and the non-acylated subclasses may perform different functions: a subclass located on the cell surface might be involved in formation of the SV40 tumor-specific transplantation antigen (TSTA), while the other one might be located at the inner side of the plasma membrane, where it might interact with cytoskeletal components. Alternatively, it is possible that large T present in the NP40-soluble fraction of the plasma membrane is not associated with this structure *in vivo*, but rather reflects cytoplasmic or nuclear large T adsorbed to plasma membranes during cell fractionation. In this case, the acylated subclass of large T would be specifically associated with the plasma membrane lamina, while also being exposed to the cell surface to act as TSTA.

Transformation of cells by SV40 requires the presence of SV40 large T (reviewed in Tooze, 1981). In contrast, expression of an intact Py large T in Py- transformed cells is not necessary: Py large T either is totally dispensable (Treisman *et al.*, 1981) or required only in a truncated form (Rassoulzadegan *et al.*, 1981; Trejo-Avila *et al.*, 1981). However, transformation by Py strictly depends on the presence of Py medium T-antigen (reviewed in Tooze, 1981). Whereas SV40 large T is a predominantly nuclear protein, Py medium T is mainly associated with plasma membranes (Ito *et al.*, 1977; Ito, 1979; Carmichael *et al.*, 1982; Schaffhausen *et al.*, 1982) and a considerable fraction of it interacts specifically with the detergent-resistant plasma membrane lamina (Schaffhausen *et al.*, 1982). A protein homologous to Py medium T has not so far been described in SV40-transformed cells. One, therefore, has to assume either that these two closely related viruses (Tooze, 1981) transform by fundamentally different pathways, or, alternatively, that functions ascribed to Py medium T are performed by SV40 large T. It is, therefore, tempting to speculate that the large T subclass described here might perform at least some functions of Py medium T.

The detergent-resistant plasma membrane component prepared here from isolated plasma membranes is, so far, only preparatively defined. However, several studies indicate that this component represents a proteinaceous lamina with some specifically retained lipids, into which certain membrane and cell surface proteins are anchored and which might provide skeletal functions for the plasma membrane (Mescher *et al.*, 1981; Ben-Ze'ev *et al.*, 1979; Carter and Hakomori, 1981). This 'membrane lamina' contains various cytoskeletal proteins (Mescher *et al.*, 1981, see Figure 2) and is associated with the cytoskeleton in *in situ* preparations, while still containing cell surface and plasma membrane proteins (Ben-Ze'ev *et al.*, 1979; Carter and Hakomori, 1981). This strongly supports the idea that this structure is linked to the bilayer structure of the plasma membrane and may provide the basis for mediating membrane signals into the interior of the cell (Ben-Ze'ev *et al.*, 1979; Carter and Hakomori, 1981). Further characterization of the detergent-resistant plasma membrane lamina described here, and the interaction of large T with this structure is currently under way.

Materials and methods

Cells

mKSA cells (a SV40-transformed BALB/c mouse tumor line) were grown in spinner culture at a denisty of $3-8 \times 10^5$ cells/ml in minimal essential medium (MEM) for suspension culture (Gibco no. F 13) supplemented with 6% (v/v) newborn calf serum (Boehringer, Mannheim, FRG).

Labeling of cells

5×10^5 mKSA cells were labeled in parallel for 4 h with either [^{35}S]-methionine or [^{3}H]palmitic acid, respectively. For labeling with [^{3}H]palmitic acid, 5 mCi of [9,10-^{3}H]palmitic acid (15 Ci/mmol, NEN) were added to 20 ml of F 13 medium supplemented with 10% tryptose phosphate broth (Gibco), 5% calf serum, 5 mM sodium pyruvate and non-essential amino acids (Gibco).

Cells were labeled with [^{35}S]methionine in 20 ml of methionine-free Eagle's medium supplemented with 5% calf serum and 1 mCi [^{35}S]methionine (1200 Ci/mmol, NEN).

Cell fractionation and subfractionation of the plasma membrane

After labeling, the cells were washed once with 10 ml KM buffer (10 mM 2-(N-morpholino)ethane sulfonic acid (MES), 10 mM NaCl, 1.5 mM $MgCl_2$, 5 mM dithiothreitol (DTT), 1 mM EGTA, pH 6.2) and resuspended in 3 ml of lysis buffer (10 mM MES, 5 mM $MgCl_2$, 5 mM DTT, 1 mM EGTA, 1% Trasylol, pH 6.5). After swelling for 10 min, the cells were homogenized in a stainless steel Dounce homogenizer until ~80% of the cells were broken. The cell lysate (homogenate) was centrifuged for 15 min at 800 *g* at 4°C in an IEC centrifuge. The supernatant was designated cytoplasmic fraction. The pellet of the low speed centrifugation was resuspended in the aqueous two-phase polymer system containing polyethylene glycol 6000 (Carbowax) and Dextran T 500 (Pharmacia) as described by Brunette and Till (1971) with the following modifications: (i) the lower phase was supplemented with 40 mg/ml Dextran T 500; (ii) $ZnCl_2$ present in the two-phase system of Brunette and Till (1971) was replaced by $MgCl_2$ to avoid unspecific precipitation of proteins; (iii) the two-phase system contained 5 mM DTT and 1 mM EGTA to prevent unspecific aggregation of proteins. Plasma membranes were collected from the

interphase of the two phases after centrifugation at 12 000 *g*, nuclei and unbroken cells were found in the pellet. A nuclear fraction was obtained from this pellet by treatment with phosphate buffered saline (PBS) containing 1% NP40.

After harvesting, plasma membranes were washed once with distilled water, resuspended in 1 ml PBS containing 5 mM DTT, 1 mM EGTA and 1% NP40 and kept for 30 min on ice. NP40-treated plasma membranes were then pelleted by centrifugation (130 000 *g* at 4°C). The NP40-insoluble material formed a tight, translucent pellet (NP40-insoluble plasma membrane fraction). The supernatant was designated NP40-soluble plasma membrane fraction. The NP40-insoluble plasma membrane fraction was resuspended by sonification in 1 ml TKS buffer (50 mM Tris, 25 mM KCl, 5 mM $MgCl_2$, 5 mM DTT, 1 mM EGTA, pH 9) containing 1% Empigen BB. Empigen BB was a generous gift from Albright and Wilson Ltd., Frankfurt, FRG. After incubating on ice for 1 h, the Empigen BB-treated NP40-insoluble plasma membrane fraction was centrifuged for 30 min at 130 000 *g*, yielding a pellet (residual plasma membrane fraction) and a supernatant (extract of the Empigen BB-treated NP40-insoluble plasma membrane fraction).

Immunoprecipitation

The following extracts were subjected to immunoprecipitation. (i) Cytoplasmic extract: the supernatant of the low speed centrifugation after cell lysis (3 ml) was centrifuged at 130 000 *g* and subsequently adjusted to 140 mM NaCl and 1% NP40 (final concentrations). (ii) Nuclear extract: the pellet of the two-phase system was resuspended in 1 ml of large T extraction buffer according to Schwyzer (1977) supplemented with 5 mM DTT and 1 mM EGTA, incubated on ice for 30 min and subjected to centrifugation at 130 000 *g* for 30 min at 4°C. (iii) NP40-soluble plasma membrane extract: isolated plasma membranes were detergent-treated as described above. The NP40 extract was then subjected to centrifugation at 130 000 *g*. (iv) Empigen BB extract of the NP40-insoluble plasma membrane fraction: the NP40-insoluble plasma membrane fraction was further extracted with Empigen BB, as described, and subjected to centrifugation at 130 000 *g*.

Extracts were incubated with 10 μl anti-SDS-T serum (Deppert and Pates, 1979) for 1 h at 4°C. Then 200 μl of protein A-Sepharose (Pharmacia, Uppsala, Sweden), preswollen in PBS, was added and the reaction mixture was incubated for 4 h at 4°C. The protein A-Sepharose containing the immunocomplexes were removed by centrifugation, and washed extensively with 0.1 M Tris buffer pH 9.0 containing 0.5 M LiCl, 1% NP40 and 1% 2-mercaptoethanol. Then the immunocomplexes were eluted from protein A-Sepharose with 50 mM NH_4HCO_3 containing 1% SDS. The eluates were lyophilized and processed for SDS-polyacrylamide electrophoresis.

Preparation of samples for gel electrophoresis

Aliquots of the cell lysate (homogenate) and the cytoplasmic fraction were diluted 1:1 with electrophoresis sample buffer (62.5 mM Tris-HCl, pH 6.8; 3% SDS and 5% 2-mercaptoethanol). The NP40-soluble plasma membrane fraction and the extract of the Empigen BB-treated insoluble plasma membrane fraction were precipitated with ice-cold trichloroacetic acid (10% v/v final concentration), washed once with ethanol and twice with acetone, air dried and dissolved in electrophoresis sample buffer. The pellet of the two-phase spin consisting of nuclei and unbroken cells was treated with PBS containing 1% NP40, pelleted and then dissolved in electrophoresis sample buffer. Pelleted plasma membranes, the NP40-insoluble plasma membrane pellet and the residual plasma membrane pellet were dissolved in electrophoresis sample buffer. Immediately after addition of sample buffer the samples were sonically treated and boiled for 3 min. The protein content of the samples was determined by the method of Lowry *et al.* (1951) and adjusted to ~1 μg/μl. Lyophylised samples of the immunoprecipitates were dissolved in 40 μl electrophoreis sample buffer omitting SDS, boiled for 3 min and subjected to SDS-polyacrylamide electrophoresis.

SDS-polyacrylamide gel electrophoresis and fluorography

The polyacrylamide gel system of Laemmli and Maizel as described by Laemmli (1970) was employed. Between 15 and 20 μg of protein in a volume of ~20 μl was applied per slot. Electrophoresis was performed at a constant current of 12 mA. After electrophoresis, the gels were prepared for fluorography as described by Bonner and Laskey (1974) and exposed for 4 days (^{35}S gel), or 3 weeks (^{3}H gel), respectively, at −70°C.

Electron microscopy

Pelleted plasma membranes, the NP40-insoluble plasma membrane fraction and the residual plasma membrane fraction after Empigen BB treatment were fixed with 3.5% glutaraldehyde in PBS overnight, washed 6 times with PBS and post-fixed for 1 h with 1% osmium tetroxide in PBS. Fixed pellets were washed with PBS, dehydrated, stained with uranylacetate in ethanol (saturated), embedded in Epon (Ladd supplies) and sectioned. Sections were stained with lead citrate and viewed in a Philips EM 301.

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