Evidence for Transmembrane Orientation of Acylated Simian Virus 40 Large T Antigen

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In mKSA cells (a simian virus 40-transformed BALB/c mouse tumor cell line), plasma membrane-associated large T antigen (large T) is found in two subfractions of the plasma membrane; a minor amount of large T is recovered from the Nonidet P-40 (NP-40)-soluble plasma membrane fraction, whereas the majority is tightly bound to a substructure of the plasma membrane, the plasma membrane lamina (PML). Only PML-associated large T is fatty acid acylated (U. Klockmann and W. Deppert, *EMBO J.* 2:1151-1157, 1983). We have analyzed whether these two forms of plasma membrane-associated large T might differ in features like cell surface expression or metabolic stability. In addition, we have asked whether one of the two large Ts might represent the hypothetical, large T-related protein T* (D. F. Mark and P. Berg, Cold Spring Harbor Symp. Quant. Biol. 44:55-62, 1979). We show that in mKSA cells grown in suspension culture, large T associated with the PML is also exposed on the cell surface. This form of large T, therefore, exhibits properties of a transmembrane protein. Large T in the NP-40-soluble plasma membrane fraction could not be labeled with radioiodine on the cell surface and, for this reason, does not seem to be oriented towards the cell surface. In contrast, when mKSA cells were grown on substratum (culture dish), we found that in these cells both NP-40-soluble large T as well as large T anchored in the PML could be cell surface iodinated. We also have analyzed the plasma membrane association of surface T antigen in mKSA cells grown in a mouse as ascites tumor. In tumor cells, only PML-bound large T is cell surface associated. We conclude that differences in extractibility of cell surface-associated large T most likely depend on cell shape and are not an artifact of cell culture. Both NP-40-soluble and PML-bound large Ts are associated with the plasma membrane in a metabolically stable fashion. Neither of the two large Ts represents T*.

Simian virus 40 (SV40)-transformed cells express a virus-specific tumor antigen, SV40 large T antigen (large T), which mediates, directly or indirectly, the pleiotropic changes involved in transformation (for a review, see reference 45). As a consequence of its interaction with different cellular structures, large T can be found in different subcellular locations; the bulk of large T is contained within the nucleus (20, 21, 31, 32, 39); a minor amount of the protein (about 1%) can be detected in the plasma membrane and on the cell surface (10, 11, 13, 15, 20, 21, 24, 27, 35-40, 47). Whereas most of the rather diverse and complex functions of large T are carried out in the nucleus (45), one activity associated with large T, i.e., the induction of SV40 tumor-specific transplantation immunity (for a review, see reference 42), is specifically restricted to the cell surface of SV40 tumor cells. SV40 tumor-specific transplantation antigen is classically defined as an antigen present on the cell surface of SV40 tumor cells (1, 9, 19), since it has the ability to induce cell-mediated rejection of a syngeneic transplant of SV40-transformed cells in preimmunized animals (42). This antigen has been shown to be closely related to large T (1, 7, 34, 43, 44).

Characterization of cell surface-associated large T has been complicated by the fact that it is present in very low amounts relative to nuclear large T and has thus far been found by immunological criteria and molecular weight to be indistinguishable from nuclear large T. We have recently analyzed the biochemical properties of this molecule. We were able to demonstrate that plasma membrane-associated

large T exhibits a specific posttranslational modification, the fatty acid acylation (20, 21), which cannot be found with nuclear large T. Therefore, plasma membrane-associated, acylated large T is a specific subclass of large T (20, 21). The plasma membrane location of large T was further analyzed by plasma membrane subfractionation experiments. Subfractionation of plasma membranes into a Nonidet P-40 (NP-40)-soluble plasma membrane fraction and a NP-40-resistant plasma membrane fraction, the plasma membrane lamina (PML), revealed that large T was present in both plasma membrane subfractions (20). Acylated large T, however, was contained exclusively within the PML fraction (20).

In this paper we have characterized the biochemically defined forms of plasma membrane-associated large Ts in more detail, since it is conceivable that these forms may vary in their association with the plasma membrane due to different functions they may perform. Either one of the two forms might be exposed on the cell surface, there being involved in the formation of SV40 tumor-specific transplantation antigen. The other form might be located at the inner side of the plasma membrane, where it might interact with cytoskeletal components. In addition, the two plasma membrane-associated forms of large T may differ in some other features, like metabolic stability, since it has been reported that cell surface-associated large T is unstable and is shed into the medium (35-37, 41). It could also be possible that either form of large T represents the large T-related protein T* postulated by Mark and Berg (29).

We here present evidence that the acylated, PML-associated form of large T spans the plasma membrane and, therefore, exhibits properties of a transmembrane protein. The specificity of its interaction with the plasma membrane,

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in regard to both orientation and posttranslational modification, suggests that this form of large T may play an important role in transformation by modulating some plasma membrane function.

MATERIALS AND METHODS

Cells. SV40-transformed BALB/c mouse tumor cells (mKSA cells) were grown in suspension (Spinner) culture at a density of 3×10^5 to 8×10^5 cells per ml in minimal essential medium for suspension culture (GIBCO no. F-13) supplemented with 5% (vol/vol) newborn-calf serum (Boehringer GmbH, Mannheim, Federal Republic of Germany) or on plates in Dulbecco modified Eagle medium supplemented with 5% fetal calf serum.

mKSA ascites tumor cells were produced by intraperitoneal injection of 10^6 mKSA cells into BALB/c mice. After 10 days, the ascites fluid was harvested, and cells were injected into another mouse. This procedure was repeated two times. Tumor cells then were directly subjected to cell surface labeling.

Labeling of cells. (i) Labeling with [35 S]methionine. mKSA cells were suspended in methionine-free Eagle medium supplemented with 5% fetal calf serum and 50 μ Ci of [35 S]methionine (specific activity, 1,200 Ci/mmol; New England Nuclear Corp., Boston, Mass.) per ml at a concentration of 1 ml of labeling medium per 5×10^6 cells.

(ii) Labeling with [3 H]palmitic acid. For labeling with [3 H]palmitic acid, cells were suspended in F-13 medium supplemented with 10% tryptose phosphate broth (GIBCO Laboratories, Grand Island, N.Y.), 5% fetal calf serum, 5 mM sodium pyruvate, the nonessential amino acids (GIBCO), and 250 μ Ci of [3 H]palmitic acid (specific activity, 15 Ci/mmol; New England Nuclear Corp.) per ml of labeling medium. After being labeled, the cells were washed once with phosphate-buffered saline (PBS) and subjected to cell fractionation (see below).

(iii) Cell surface labeling with sodium [125 I]iodide. Cells were washed three times with PBS (pH 7.0) containing 0.5 mM MgCl_2 and 1 mM CaCl_2 to remove serum. Carrier-free Na^{125}I (specific activity, 2,000 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) was added at a volume of 500 μ Ci per 10^7 cells in 1 ml of PBS containing CaCl_2 , MgCl_2 , and 5 mM glucose. The reaction was started by the addition of lactoperoxidase and glucose oxidase (Boehringer) to final concentrations of 50 μ g/ml and 1 U/ml, respectively. After a 10-min incubation at room temperature, the reaction was stopped by the addition of cold PBS with NaI replacing NaCl (PBS_1). After being washed twice with PBS_1 , the cells were subjected to cell fractionation. For pulse-chase experiments, the labeling medium was removed after the labeling period; the cells were split into two parts and washed once with PBS. One part (pulse) was immediately subjected to cell fractionation. The remaining cells were diluted with medium (final concentration, 5×10^5 cells per ml) and kept in this medium for the time indicated in the figure legends. These cells (chase) were then washed once with PBS and subjected to cell fractionation.

Cell fractionation and subfractionation of plasma membranes. Fractionation of mKSA suspension cells was performed essentially as described by Klockmann and Deppert (20). Plasma membranes were prepared in the aqueous two-phase polymer system containing polyethylene glycol 6000 and dextran T 500 as described by Brunette and Till (5) with the modifications outlined previously (20). Isolated plasma membranes were resuspended in 1 ml of PBS con-

taining 5 mM dithiothreitol, 1 mM ethylene glycol-bis(β -aminoethylether)- N,N,N',N' -tetraacetic acid (EGTA), and 1% NP-40 and kept for 30 min on ice. NP-40-treated plasma membranes were then pelleted by centrifugation ($130,000 \times g$ at 4°C). The NP-40-insoluble material formed a tight, translucent pellet and was designated the PML. The supernatant was designated NP-40-soluble plasma membrane fraction.

Detergent lysis of mKSA cells. After cell surface iodination, mKSA suspension cells were washed twice with PBS_1 . NP-40 extraction buffer (PBS_1 [pH 7.0], 1% NP-40, 1% Trasylol) was gently pipetted onto the cell pellet, and cells were incubated for 15 min on ice. The NP-40-insoluble cellular structures were pelleted ($130,000 \times g$, 4°C), and the supernatant containing the NP-40-soluble material was removed. The structures were further treated with Empigen BB extraction buffer (25 mM KCl, 5 mM MgCl_2 , 50 mM Tris hydrochloride [pH 9.0], 1% Empigen BB, 1% dithiothreitol, 1% Trasylol) and kept for 30 min on ice. After centrifugation, the supernatant containing the Empigen BB-solubilized material was removed.

mKSA cells grown in monolayer were extracted as follows. The cells were washed twice with PBS_1 . NP-40 extraction buffer was added, and the plates were gently tilted from side to side during the extraction. After 15 min on ice, the supernatant was removed, and cellular structures remaining on the plates were washed once with PBS_1 and further incubated with Empigen BB extraction buffer for 30 min on ice.

NP-40 extracts and Empigen BB extracts were subjected to immunoprecipitation.

Immunoprecipitation and preparation of samples for gel electrophoresis. Extracts were prepared for immunoprecipitation as described previously (20, 22). Extracts were incubated with 10 μ l of anti-sodium dodecyl sulfate (SDS)-T serum (12) for 1 h at 4°C . Then, 200 μ l of protein A-Sepharose (Pharmacia Fine Chemicals, Piscataway, N.J.), preswollen in PBS, was added, and the reaction mixture was incubated for 4 h at 4°C . The protein A-Sepharose containing the immunocomplexes was removed by centrifugation and washed extensively with 0.1 M Tris buffer (pH 9.0) containing 0.5 M LiCl, 1% NP-40, and 1% 2-mercaptoethanol. The immunocomplexes then were eluted from protein A-Sepharose with 50 mM NH_4HCO_3 containing 1% SDS. The eluates were lyophilized, dissolved in 40 μ l of electrophoresis sample buffer without SDS, boiled for 3 min, and subjected to SDS-polyacrylamide gel electrophoresis. Samples of cell fractions obtained during cell fractionation were prepared for gel electrophoresis as described previously (20). The protein content of the samples was determined by the method of Lowry et al. (28) and adjusted to $\sim 1 \mu\text{g}/\mu\text{l}$.

SDS-polyacrylamide gel electrophoresis and fluorography. The polyacrylamide gel system of Laemmli and Maizel as described by Laemmli (23) was employed. About 20 μg of protein in a volume of $\sim 20 \mu\text{l}$ was applied per slot. Electrophoresis was performed at a constant current of 12 mA. After electrophoresis, gels containing [35 S]methionine- or [3 H]palmitic acid-labeled proteins were prepared for fluorography as described by Bonner and Laskey (4) and exposed for the times indicated in the figure legends.

RESULTS

Characterization of plasma membrane-associated forms of large T. All experiments were performed with the SV40-transformed BALB/c mouse tumor cell line mKSA cells grown in suspension culture. After being radioactively la-

beled, cells were fractionated into nuclear, cytoplasmic, and plasma membrane fractions as described previously (20, 22). The plasma membrane fraction then was subfractionated; isolated plasma membranes were treated first with NP-40, resulting in the preparation of the PML (20). The PML was further treated with the zwitterionic detergent Empigen BB, which almost completely dissolves this substructure (20). Figure 1A shows an analysis of plasma membrane subfractions for the presence of large T after [35 S]methionine labeling of mKSA suspension cells. Only about one-third of large T associated with isolated plasma membranes was obtained from the NP-40-soluble plasma membrane fraction (Fig. 1A, NP-40 PM). The residual plasma membrane-associated large T (about two-thirds of the total) was tightly bound to the NP-40-resistant PML, from which it was released by treatment with Empigen BB (Fig. 1A, PML) as reported previously (20, 22). Only large T associated with the PML contained covalently bound fatty acid (Fig. 1B, PML). Large T in the NP-40-soluble plasma membrane fraction could not be labeled with [3 H]palmitic acid (Fig. 1B, NP40 PM).

We have addressed the question of whether either one of the two plasma membrane-associated forms of large T might represent the hypothetical protein T* postulated by Mark and Berg (29). These authors have reported the existence of an additional species of a differently spliced SV40 RNA, which should code for a third early SV40 protein related in structure to large T. T* would differ in length from large T by only two amino acids. The presumed splice, however, should cause a striking difference in the 70 carboxy-terminal amino acids, resulting in a rather hydrophobic carboxy terminus for the putative T* protein. The altered carboxy terminus contained in T*, however, should not be recog-

FIG. 1. SDS-polyacrylamide gel fluorograms of SV40 large T in plasma membrane subfractions of mKSA suspension cells. Parallel cultures of [35 S]methionine (A and C)- or [3 H]palmitic acid (B)-labeled cells were subfractionated. The NP-40-soluble plasma membrane fraction (NP40 PM) and the Empigen BB extract of the PML fraction (PML) were immunoprecipitated with anti-SDS-T serum (A and B) or with an antiserum directed against the carboxy terminus of large T (C). Samples of the immunoprecipitates were applied to an 11.5% acrylamide gel and run for 6 h at 12 mA. 35 S gels were exposed for 3 days, and the 3 H gel was exposed for 21 days.

FIG. 2. SDS-polyacrylamide gel autoradiograms of distribution of large T in subcellular fractions of cell surface-iodinated mKSA suspension cells. (A) mKSA cells grown in suspension culture were cell surface iodinated and subfractionated. Extracts were prepared and immunoprecipitated as described in Materials and Methods. Large T was immunoprecipitated from the cytoplasmic fraction (C), the nuclear fraction (N), the NP-40-soluble plasma membrane fraction (NP40 PM), and the Empigen BB extract of the PML fraction (PML). (B) mKSA cells grown in suspension culture were cell surface iodinated and directly extracted with NP-40 (lane a) followed by Empigen BB (lane b), and extracts were analyzed by immunoprecipitation for the presence of iodinated large T.

nized by an antibody directed against the carboxy-terminal peptide of authentic large T (46). We have used such an antibody and found that it reacted with both large T in the NP-40-soluble plasma membrane fraction (Fig. 1C, NP40 PM) and large T solubilized from the PML (Fig. 1C, PML) in a manner identical to the reaction with anti-SDS-T serum (Fig. 1A). In addition, we have also used an antibody directed against a synthetic oligopeptide corresponding to the proposed carboxy-terminal region of T* (kindly provided by Gernot Walter, University of California, San Diego) to precipitate the plasma membrane-associated forms of large T. This antibody, however, did not react with either large T in the NP-40-soluble plasma membrane fraction or with PML-associated large T (data not shown). We therefore conclude that both forms of plasma membrane-associated large T are identical in their amino acid sequences to nuclear large T.

Cell-surface localization of plasma membrane-associated forms of large T. To determine whether PML-associated large T or large T in the NP-40-soluble plasma membrane fraction is exposed on the cell surface, we used lactoperoxidase-catalyzed cell surface iodination for labeling the cells before cell fractionation. mKSA suspension cells were surface labeled for 10 min with 125 I as described in Materials and Methods. After being labeled, the cells were fractionated as described above, and cellular subfractions were analyzed for the presence of 125 I-labeled large T. Figure 2A shows the distribution of 125 I-labeled large T in the different cellular subfractions. Neither the cytoplasmic nor the nuclear fraction contained 125 I-labeled large T (Fig. 2A, C, and N), demonstrating that lactoperoxidase-catalyzed iodination of

FIG. 3. SDS-polyacrylamide gel fluorograms of distribution of large T in different subcellular fractions of mKSA suspension cells after pulse and pulse-chase labeling with ^{125}I or [^{35}S]methionine. Parallel cultures of mKSA suspension cells were either cell surface iodinated (A) or labeled with [^{35}S]methionine (B). After being labeled, cells were split into two parts; one part (pulse [p]) was immediately subjected to cell fractionation, and the remaining cells were diluted with medium, incubated for 2 (A) or 4 (B) h, and subsequently fractionated (chase [c]). Extracts were immunoprecipitated with anti-SDS-T serum. Samples of the immunoprecipitates were applied to an 11.5% acrylamide gel and run for 6 h at 12 mA. Large T was immunoprecipitated from the cytoplasmic fraction (C), the nuclear fraction (N), the NP-40-soluble plasma membrane fraction (NP40 PM), and the Empigen BB extract of the PML fraction (PML).

proteins was confined to the cell surface. Large T in the NP-40-soluble plasma membrane fraction was not labeled and, therefore, does not seem to be oriented towards the cell surface (Fig. 2A, NP40 PM). PML-associated large T could be labeled with ^{125}I and, for this reason, is exposed on the cell surface (Fig. 2A, PML).

To exclude the possibility that large T exposed on the cell surface might have attached artificially to the PML during plasma membrane preparation, we extracted ^{125}I -labeled large T directly from surface-iodinated cells. mKSA suspension cells were surface labeled with ^{125}I for 10 min. After being labeled, the cells were washed once with PBS_i (see Materials and Methods) and then incubated with NP-40 extraction buffer. The NP-40 extract of the cells was removed, and the remaining NP-40-insoluble cellular structures were treated with Empigen BB extraction buffer. The NP-40 and Empigen BB extracts of the cells were analyzed for the presence of iodinated large T. The NP-40 extract of surface-iodinated mKSA suspension cells contained only a very small amount of ^{125}I -labeled large T (Fig. 2B, lane a). The bulk of iodinated large T was obtained from the Empigen BB extract of the NP-40-insoluble cellular structures (Fig. 2B, lane b). We conclude that *in vivo* large T exposed on the cell surface of mKSA suspension cells is also tightly associated with an NP-40-insoluble structure.

Metabolic stability of plasma membrane-associated forms of large T. It has been reported previously that large T exposed on the cell surface of SV40-transformed cells is metabolically unstable (35–37, 41). After surface iodination of cells followed by a short incubation in culture medium, ^{125}I -labeled large T could no longer be detected in association with the cells (35–37, 41). Since ^{125}I -labeled large T could be immunoprecipitated from the medium of the chased cultures, these authors concluded that surface-associated large T had been shed into the culture supernatant (41). In our

experiments, cell surface-associated large T was tightly bound to the PML. It might therefore be expected that this surface T is associated with the plasma membrane in a metabolically stable fashion. To analyze whether PML-associated large T in our cells was metabolically stable, we investigated its stability after pulse and pulse-chase labeling of mKSA suspension cells with ^{125}I . mKSA suspension cells were surface iodinated for 10 min (pulse). A sample of the pulse-labeled cells was held in growth medium for 2 h (chase). Both pulse- and pulse-chase-labeled cells were fractionated as described above, and fractions were analyzed for the presence of ^{125}I -labeled large T. Figure 3A shows ^{125}I -labeled large T in subcellular fractions after pulse and pulse-chase labeling of mKSA suspension cells with ^{125}I . ^{125}I -labeled large T was immunoprecipitated only from the Empigen BB extract of the PML of pulse-labeled cells (Fig. 3A, PML, lane p) but not from the Empigen BB extract of the PML of pulse-chase-labeled cells (Fig. 3A, PML, lane c). Thus, ^{125}I -labeled large T seems to exhibit a very short half-life.

However, ^{125}I labeling of cell surface components is a very drastic process. Covalent modification of proteins and lipids with iodine may be highly toxic for the cells, thus leading to an increased turnover of chemically altered membrane constituents. In accordance with this idea are reports that describe how chemical alteration of cell surface components resulted in a reduced metabolic stability of cell surface proteins (8, 18). We have therefore analyzed the effect of cell surface iodination on the metabolic stability of cell surface proteins by comparing the metabolic stability of plasma membrane proteins of mKSA cells after ^{125}I or [^{35}S]methionine labeling. mKSA suspension cells were surface iodinated for 10 min (pulse). A sample of the pulse-labeled cells was held in normal growth medium for 2 h (chase). Another batch of mKSA cells was labeled with [^{35}S]methionine for 30

min (pulse). A sample of these cells was held for 2 h in growth medium containing an excess of unlabeled methionine (chase). Both pulse- and pulse-chase-labeled cells were fractionated as described above. Figure 4 shows the PML fraction and the NP-40 extract of the plasma membrane after pulse (p) and pulse-chase (c) labeling of mKSA suspension cells with ^{125}I (Fig. 4A) or [^{35}S]methionine (Fig. 4B). Equal amounts of protein were analyzed in each slot of the gels shown in Fig. 4. Figure 4A reveals a distinct difference in the intensity of the ^{125}I label of cell surface proteins both in the PML fraction and in the NP-40-soluble plasma membrane fraction after pulse and pulse-chase labeling. In contrast, no significant change in [^{35}S]methionine-labeled membrane proteins was seen between pulse- and pulse-chase-labeled cells. This fact suggests that ^{125}I -labeled membrane proteins, in contrast to membrane proteins labeled with [^{35}S]methionine, exhibit a reduced metabolic stability. This conclusion implies that it may be difficult, if not impossible, to determine the metabolic stability of cell surface proteins after cell surface iodination. We therefore analyzed the metabolic stability of plasma membrane-associated large T after metabolic labeling with [^{35}S]methionine. mKSA cells were labeled for 30 min with [^{35}S]methionine (pulse). A sample of the pulse-labeled cells was held for 4 h in normal growth medium containing an excess of unlabeled methionine (chase). Both pulse- and pulse-chase-labeled cells were fractionated as described. The cytoplasmic fraction, the extract of the nuclear fraction, the NP-40-soluble plasma membrane fraction, and the Empigen BB extract of the PML fraction were analyzed for the presence of [^{35}S]methionine-labeled large T. Figure 3B shows the relative amounts of large T in the different subcellular fractions after pulse and pulse-chase labeling of mKSA suspension cells with [^{35}S]methionine. The amounts of [^{35}S]methionine-labeled large T in the PML and the NP-40-soluble plasma membrane fractions did not differ significantly after pulse and pulse-chase labeling of the cells. Therefore, we conclude that both forms of plasma membrane-associated large T are metabolically stable.

Distribution of cell surface-associated large T in detergent extracts of mKSA cells grown on substratum. Plasma membrane subfractionation as well as detergent lysis of mKSA suspension cells have shown that ^{125}I -labeled large T is almost quantitatively associated with the PML and could not be extracted with nonionic detergents like NP-40. However, it has been reported that iodinated large T is easily extracted from SV40-transformed cells with NP-40 containing buffers (35–38, 41). These data seem to contradict our data, since NP-40 detergent extractions were also performed with the mKSA cell line used by us. A significant difference in these experiments, however, was that they were carried out with mKSA cells attached to a substratum (culture plate) and not with mKSA cells grown in suspension culture.

To analyze whether the solubility of surface T antigen differs depending on different growth conditions of the cells (growth on a substratum versus growth in suspension culture), we investigated the extractability of iodinated large T from mKSA cells grown on plates. These cells were surface iodinated for 10 min. The iodination buffer was removed, and the cells were washed twice with PBS_I (see Materials and Methods) and then incubated in PBS_I plus 1% NP-40 for 15 min on ice. The NP-40 extract was removed, and the remaining cellular structures on the culture dish were reextracted with the same NP-40 containing buffer. Afterwards, the NP-40-resistant cellular structures were further treated with Empigen BB extraction buffer (see Materials

FIG. 4. SDS-polyacrylamide gel fluorograms of polypeptide patterns of plasma membrane subfractions of mKSA suspension cells after pulse and pulse-chase labeling with ^{125}I or [^{35}S]methionine. Parallel cultures of mKSA suspension cells were either cell surface iodinated (A) or labeled with [^{35}S]methionine (B). After being labeled, the cells were split into two parts; one part (pulse [p]) was immediately subjected to cell fractionation, and the remaining cells were diluted with medium, incubated for 2 h, and subsequently fractionated (chase [c]). Isolated plasma membranes were subfractionated into the NP-40-soluble plasma membrane (NP40 PM) and PML fractions. Approximately 15 μg of each fraction was applied to an 11.5% SDS-polyacrylamide gel and run for 6 h at 12 mA. The ^{35}S gel was processed for fluorography. Both gels were exposed for 2 days.

and Methods). The three NP-40 extracts and the Empigen BB extract of iodinated mKSA cells were analyzed for the presence of iodinated large T. The result is shown in Fig. 5. The majority of ^{125}I -labeled large T (about two-thirds) was obtained in the first NP-40 extract of the cells. No further ^{125}I -labeled large T was solubilized with two subsequent NP-40 extractions. About one-third of the ^{125}I -labeled large T was recovered from the Empigen BB extract. Thus, the distribution of ^{125}I -labeled large T in detergent extracts of mKSA cells grown on plates differs from that of mKSA cells grown in suspension culture.

Distribution of cell surface-associated large T in detergent extracts of mKSA cells taken from ascites tumors. Analysis of the plasma membrane association of large T in mKSA cells kept under different growth conditions resulted in differences in the extractability of cell surface-associated large T. Since it is difficult to decide which result might be influenced by culture conditions, we analyzed the plasma membrane association of surface T antigen in mKSA cells directly taken from an ascites tumor in a mouse. mKSA tumor cells were harvested from the ascites fluid of a mKSA tumor-bearing mouse (see Materials and Methods), washed two times with medium and once with PBS_I , and then surface iodinated for 10 min. After being labeled, the cells were first extracted with NP-40. The NP-40 extract of the cells was removed, and the remaining NP-40-insoluble cellular structures were extracted with Empigen BB. The NP-40 and Empigen BB extracts of the cells were analyzed for the presence of iodinated large T. The NP-40 extract contained a very small

FIG. 5. SDS-polyacrylamide gel autoradiogram of distribution of ^{125}I -labeled large T in detergent extracts of cell surface-iodinated mKSA cells grown on plates. mKSA cells grown on plates were surface iodinated and subsequently detergent extracted in situ as described in the text. Extracts were immunoprecipitated with anti-SDS-T serum. A sample of the immunoprecipitates was applied to an 11.5% SDS-polyacrylamide gel and run for 6 h at 12 mA. Large T was immunoprecipitated from NP-40 extracts 1 (lane a), 2 (lane b), and 3 (lane c) and from the Empigen BB extract of the NP-40-insoluble residual cellular structures (lane d).

amount of iodinated large T (Fig. 6, lane a). The major amount of iodinated large T was obtained in the Empigen BB extract of the NP-40-insoluble cellular structures (Fig. 6, lane b). Thus, cell surface-associated large T in mKSA cells taken from ascites tumor, like cell surface-associated large T in mKSA cells grown in suspension culture, is tightly associated with an NP-40-insoluble plasma membrane substructure.

DISCUSSION

In this study we have analyzed various features of plasma membrane-associated large T in more detail. Acylated large T is tightly bound to a detergent-resistant substructure of the plasma membrane, the PML (20). The PML has been shown to directly underly the lipid bilayer of the plasma membrane (2, 6, 30), thereby connecting the plasma membrane with the cytoskeleton (2, 6, 30). Therefore, we have considered the possibility that acylated large T is associated with the cytoplasmic face of the plasma membrane. However, cell surface iodination studies revealed that acylated, PML-associated large T can be labeled with lactoperoxidase and ^{125}I on the cell surface and thus is exposed. This fact strongly suggests that acylated large T spans the plasma membrane and, therefore, is a transmembrane protein.

Although large T traverses the lipid bilayer of the plasma membrane, this protein does not exhibit the features of a

typical transmembrane protein. Most bona fide transmembrane proteins interact with the lipid bilayer by extended sequences of hydrophobic amino acids. However, the amino acid sequence of large T derived from the nucleotide sequence of SV40 DNA (14, 33) lacks an extensive hydrophobic stretch of 20 or more amino acids thought to be necessary for traversal of the lipid bilayer. Thus, it is difficult to imagine a mechanism for the insertion of large T into the membrane as an integral membrane protein, complying with the trigger (48) or the signal sequence (3) hypothesis. Recently, we have pointed out an amino acid sequence within the carboxy-terminal region of large T (Ile₅₆₈-Tyr₅₈₂) which might be important for its association with the plasma membrane (22). However, whether this hydrophobic sequence really is involved in membrane anchoring of large T awaits further analysis. Alternatively, it is conceivable that fatty acid(s) bound to large T may participate in membrane anchoring of this molecule. However, we have found that a small carboxy-terminal fragment of large T, the SV40 28,000-molecular-weight protein encoded by the nondefective adenovirus type 2-SV40 hybrid virus Ad2⁺ND1, does not carry any attached fatty acid but is associated with the plasma membrane in a metabolically stable fashion, providing an argument against the necessity of acylation of large T for membrane association (22). The 28,000-molecular-weight

FIG. 6. SDS-polyacrylamide gel autoradiogram of distribution of ^{125}I -labeled large T in detergent extracts of cell surface-iodinated mKSA cells grown in ascites. mKSA cells grown in a mouse as an ascites tumor were cell surface iodinated and directly extracted with NP-40 (lane a) followed by Empigen BB (lane b). Extracts were analyzed by immunoprecipitation for the presence of iodinated large T.

protein, however, was not bound to the PML, whereas larger fragments of large T which contained covalently bound fatty acid, as well as acylated large T itself, were tightly bound to the PML (22). This finding suggested that fatty acid acylation correlates with PML association. Therefore, it is possible that fatty acid(s) bound to large T are involved in the association of the protein with the PML rather than in its insertion into the lipid bilayer. However, in an analysis of the molecular features mediating the plasma membrane association of large T, it is important to realize that there might be other not-yet-defined features which might lead to a transmembrane orientation of a protein. In this context, Williams and Gagnon (49) recently described a transmembrane glycoprotein isolated from murine T-lymphoma cells, which, like large T, does not exhibit the features of a typical transmembrane protein.

Soule et al. (41) reported that plasma membrane-associated large T labeled on the cell surface with ^{125}I and lactoperoxidase is metabolically unstable and is shed into the medium. In accordance with their data, we also found that after radioiodination, cell surface-associated labeled large T exhibits a very short half-life. Iodination, however, results in a chemical modification of cell surface components, especially of unsaturated fatty acids in membrane lipids. This method, therefore, is considered to have drastic effects on the cells, which may influence the turnover of membrane constituents. In this study we demonstrate that iodination of cells leads to an enhanced turnover of cell surface proteins. An enhanced turnover of chemically altered membrane constituents had been shown also by Kapeller et al. (18) and Cone et al. (8). Due to the caveats in interpreting radioiodination experiments, we have analyzed the metabolic stability of PML-bound large T after metabolic labeling with [^{35}S]methionine. We found that large T then was stably associated with the plasma membrane for the time investigated (30-min pulse, 4-h chase). This result demonstrates that at least the majority of PML-associated large T is metabolically stable. Since plasma membrane-associated large T constitutes only a minor fraction of total large T, it was important to be sure that metabolically labeled plasma membrane-associated large T was not contaminated by even low levels of nuclear large T. The following controls strongly argue against a contamination of plasma membrane-associated large T by nuclear large T during cell fractionation. (i) Dounce homogenization of cells did not lead to damage of the nuclei. Nuclear integrity was largely preserved, judged by the negligible release of nuclear large T into the cytoplasm (Fig. 3B, lane C). (ii) After separation of nuclei from plasma membranes, isolated plasma membranes were obtained largely free from contaminating nuclei, as monitored by phase-contrast microscopy. (iii) For preparation of the PML, isolated plasma membranes were extracted with NP-40; contaminations by nuclear large T should have been removed by this procedure. We therefore conclude that acylated, PML-associated large T is a transmembrane protein, associated with the plasma membrane in a metabolically stable fashion.

Because of the unique characteristics of PML-associated large T, it is tempting to speculate that it exhibits important biological functions. Interaction of large T with the plasma membrane might affect regulatory mechanisms of the plasma membrane, resulting in an altered growth control and drastically altered cell-cell interactions. The simultaneous exposure of certain domains of this molecule on the cell surface would allow the involvement of the protein in the formation of SV40 tumor-specific transplantation antigen. Suggestive

evidence for a role in transformation for plasma membrane-associated large T has recently been provided by Lanford and Butel (25, 26) and Kalderon et al. (16, 17). These workers have analyzed a mutant of large T which has the peculiar property of not being transported into the nucleus, but instead accumulates in the cytoplasm and on the cell surface. This mutant has the ability to transform established cell lines with the same efficiency as wild-type large T.

In mKSA cells grown in suspension culture, only PML-associated large T seems to be exposed on the cell surface, since neither by subfractionation of isolated plasma membranes nor by differential extraction of unfractionated cells with NP-40 and Empigen BB were we able to detect significant amounts of NP-40-soluble, radioiodinated large T. In contrast, we recovered a significant amount of NP-40-soluble surface-associated large T from mKSA cells grown on substratum. This obvious difference in extractibility of cell surface-associated large T in cells grown in suspension culture when compared with cells grown on substratum may depend on different growth conditions. Since cells attached to a substratum differ in their cytoskeletal architecture from cells grown in suspension culture, it is conceivable that large T may be anchored differently in the plasma membrane of these cells. However, it is important to note that mKSA cells grown on plates also contained a considerable amount of PML-associated large T exposed to the cell surface in addition to NP-40-soluble large T. We cannot exclude the possibility that NP-40-soluble, cell surface-associated large T also exists in suspension cells, but that there it is not accessible for radioiodination. In fact, subfractionation of isolated plasma membranes yields a minor amount of NP-40-soluble large T, the origin of which is difficult to determine since it lacks any specificity marker like acylation or ability to be cell surface iodinated. It is therefore possible that mKSA suspension cells do not contain surface-associated, NP-40-soluble large T and that NP-40-soluble cell surface large T represents an additional form of large T present only in mKSA cells grown on substratum. Since NP-40-soluble cell surface-associated large T is also not detectable in mKSA ascites tumor cells, it seems that it is dispensable at least in these tumor cells.

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