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VIROLOGY 143, 127–142 (1985)

Identification and Characterization of a Mouse Mammary Tumor Virus Protein Uniquely Expressed on the Surface of BALB/cV Mammary Tumor Cells

BETTY L. SLAGLE AND JANET S. BUTEL[1]

Department of Virology and Epidemiology, Baylor College of Medicine, Houston, Texas 77030

Received October 1, 1984; accepted January 3, 1985

A unique subline of BALB/c mice, designated BALB/cV, exhibits an intermediate mammary tumor incidence (47%) and harbors a distinct milk-transmitted mouse mammary tumor virus (MMTV). The BALB/cV subline was used to study the molecular basis of potential virus-host interactions involving cell surface-expressed MMTV proteins. Cell surface iodination identified virus-specific proteins expressed on BALB/cV primary mammary tumor cells grown in culture. In contrast to (C3H)MMTV-producing cell lines which expressed MMTV gp52, BALB/cV tumor cells lacked gp52 and expressed instead a 68K, *env*-related protein. The $68K^{env}$ protein was also detected on the surface of metabolically labeled BALB/cV tumor cells by an external immunoprecipitation technique. The expression of $68K^{env}$ was restricted to mammary tissues of BALB/cV mice that also expressed other MMTV proteins. Biochemical analysis established that $68K^{env}$ was not modified by N-linked glycosylation. ^{125}I-labeled $68K^{env}$ was rapidly released into the media of tumor cell cultures and was recovered both in the form of a soluble protein and in a 100,000 *g* pellet. The biologic function of this cell surface-expressed viral protein remains unknown.

INTRODUCTION

BALB/c mice are commonly used as a model system for the study of mammary tumorigenesis because they exhibit a low incidence of spontaneous mammary tumors, they lack the exogenous milk-transmitted mouse mammary tumor virus (MMTV), and they are susceptible to tumor induction by a variety of exogenous factors (Michalides *et al.*, 1979; Pauley *et al.*, 1979; Butel *et al.*, 1981; Bentvelzen, 1982). The endogenously transmitted MMTV sequences of BALB/c mice are organized into three proviruses, designated units I, II, and III (Cohen *et al.*, 1979; Cohen and Varmus, 1980), or Mtv-6, -8, and -9, respectively (Traina *et al.*, 1981). The expression of the BALB/c endogenous proviruses is generally limited to 3′ long terminal repeat (LTR) sequences (Dudley *et al.*, 1978; Wheeler *et al.*, 1983; van Ooyen *et al.*, 1983; Breznik *et al.*, 1984).

A unique subline of BALB/c mice, designated BALB/cV, has recently been described (Drohan *et al.*, 1981; Slagle *et al.*, 1984). Whereas BALB/cV mice have a spontaneous mammary tumor incidence of 47%, the parental BALB/cCrlMed mice from which the BALB/cV subline was derived maintain a tumor incidence of <1%. The milk-transmitted (BALB/cV)-MMTV shares group-specific antigenic determinants with (C3H)MMTV on each of the virus structural proteins (Slagle *et al.*, 1984), but it reportedly can be distinguished from all known strains of MMTV by both immunological and molecular criteria (Drohan *et al.*, 1981). The origin of the BALB/cV isolate remains unknown. Although it could have originated by infection of a BALB/c mouse with a unique

[1] Author to whom reprint requests should be addressed.

0042-6822/85 $3.00

exogenous variant, the possibility also exists that it may represent an activation of one of the BALB/c endogenous proviruses. Expression of endogenous MMTV has been documented in C3H mice (DeOme *et al.*, 1959; Van Nie and Verstraeten, 1975; Vacquier *et al.*, 1981; Puma *et al.*, 1982).

MMTV-related antigens have been detected at the surface of mammary tumor cells in several mouse strains (for a review, see Bentvelzen and Hilgers, 1980). The expression of viral-specific antigens at the surface of virus-infected or -transformed cells may be important for several reasons. Surface-associated structural proteins are frequently involved in the maturation pathways of viruses which bud from the cell. Additionally, surface-expressed viral antigens are more likely to be detected by host immune surveillance systems than are viral proteins localized inside the cell. Thus, immunization strategies would be most logically directed against those exposed antigens. Finally, the possibility exists that virus-specific cell surface antigens might be shed from the cell and serve as tumor-blocking factors, with subsequent effects on the host immune regulation of growing tumor cells.

We have used the BALB/cV subline of mice to investigate the molecular basis of potential virus-host interactions involving surface-associated viral proteins in the mammary system. We first used cell surface iodination to identify BALB/cV proteins expressed at the surface of tumor cells in primary cultures. In contrast to C3H-producing cell lines, BALB/cV tumor cells lacked detectable levels of cell surface gp52 and expressed instead a 68K, *env*-related protein. We then examined the basis for the aberrant cell surface localization of this protein. $68K^{env}$ does not appear to be modified by glycosylation and was highly unstable at the cell surface. Labeled $68K^{env}$ shed into the media was present both as a soluble protein and in a form that could be pelleted by high-speed centrifugation. Although the biologic role of BALB/cV surface $68K^{env}$ remains obscure, several intriguing possibilities are discussed.

MATERIALS AND METHODS

Viruses and cells. Concentrated (C3H)-MMTV (Lot No. P-1033) was obtained from the Biological Carcinogenesis Branch, Division of Cancer Cause and Prevention, National Cancer Institute.

Mm5mt/c1 cells (Owens and Hackett, 1972; Fine *et al.*, 1974) and H-1 cells (Scolnick *et al.*, 1976) produce (C3H)MMTV, while MTV-L cells from a BALB/cV animal (Butel *et al.*, 1977) are virus free. The cells were cultivated in Dulbecco's minimum essential medium (D-MEM) containing 10% heat-inactivated fetal bovine serum (FBS; Grand Island Biological Co., Grand Island, N. Y.), 0.1 μg/ml gentamicin sulfate, 10 μg/ml insulin (Sigma Chemical Co., St. Louis, Mo.), 2 μg/ml dexamethasone (Sigma), and 0.3% sodium bicarbonate in a humidified atmosphere of 10% CO_2 at 37°.

Antisera. Antisera against detergent-disrupted (C3H)MMTV [anti(C3H)MMTV^d], affinity-purified (C3H)MMTV gp52/gp36 (anti-gp52/gp36), and gel-purified (C3H)MMTV p28 (anti-p28) were prepared in rabbits. The specificities of these antisera have been detailed previously (Slagle *et al.*, 1984). Adsorption experiments have demonstrated that the anti-gp52/gp36 serum reacts specifically with MMTV glycoproteins and envelope-related precursors and does not react with normal cell proteins of BALB/c mammary tissue (Slagle *et al.*, 1985).

Mice. All mice were from a conventional closed mouse colony housed in the Department of Cell Biology, Baylor College of Medicine. The BALB/cV substrain was derived from a BALB/cCrlMed mouse, as described (Drohan *et al.*, 1981; Slagle *et al.*, 1984). BALB/cCrlMed mice were used for the transplantation of Cv-2 HAN outgrowths as previously described (Slagle *et al.*, 1984).

Establishment of primary tumor cell cultures. Primary cell cultures of BALB/cV tumor cells were established as reported (Slagle *et al.*, 1984) and grown in the media described above. Only primary tumors arising spontaneously from transplants of the Cv-2 HAN outgrowth line (Slagle *et al.*, 1984) were analyzed in these

experiments, with the exception of a serially transplanted BALB/cV tumor included as a control in Fig. 5.

Lactoperoxidase-catalyzed cell surface iodination. Intact cell monolayers were iodinated according to the procedure of Soule *et al.* (1982). Previous studies from our laboratory have established the surface specificity of this iodination procedure (Soule *et al.*, 1982; Santos and Butel, 1982; Lanford and Butel, 1982). Cells grown in 100-mm plates were rinsed three times with Tris-buffered saline (TBS; 2 m*M* Tris, pH 7.4, 140 m*M* NaCl, 5 m*M* KCl, 0.4 m*M* Na_2HPO_4, 6 m*M* dextrose, 0.5 m*M* $MgCl_2$, and 0.7 m*M* $CaCl_2$), and a fourth time with Dulbecco's phosphate-buffered saline (D-PBS; Dulbecco and Vogt, 1954). One milliliter of D-PBS containing 1 mCi ^{125}I-Na (>350 mCi/ml; Amersham, Arlington Heights, Ill.) and 28 μl of a 1 mg/ml solution of freshly prepared lactoperoxidase (Calbiochem-Behring Corp., La Jolla, Calif.) were added per plate. Each plate then received 28 μl of a 10^{-4} dilution of 30% H_2O_2 (Fisher, Dallas, Tex.) at 0, 2, 4, and 6 min, with gentle rotation of plates during the 2-min intervals. At the end of the 8-min labeling period, the D-PBS/^{125}I was removed and the cell monolayers either were rinsed in cold TBS and extracted or were rinsed with warm TBS, serum-free media added, and the cells incubated at 37° for a chase period before extraction.

Analysis of iodinated protein(s) shed into culture fluid. Media collected from iodinated cell monolayers after a 15-min chase period were clarified by centrifugation at 15,000 rpm for 30 min. The supernatant was recovered and subjected to a second centrifugation for 1 hr at 100,000 *g* through a 30% sucrose cushion. The supernatant of the high-speed centrifugation was immunoprecipitated using rabbit antisera. The pellet was dissolved in extraction buffer (EB) and then immunoprecipitated. EB consisted of 50 m*M* Tris-HCl, pH 8.0, 100 m*M* NaCl, 1% NP-40, and 1% Trasylol (Mobay Chemical Co., New York, N. Y.).

Immunoprecipitation of labeled extracts. Labeled cells were extracted in EB and immunoprecipitated as previously described (Lanford and Butel, 1979; Slagle *et al.*, 1984). Immune complexes were dissociated using gel disruption buffer (0.5 *M* Tris-HCl, pH 6.8, 2% SDS, 2% 2-mercaptoethanol, 10% glycerol, and 0.002% bromophenol blue) and then analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

SDS-PAGE. Discontinuous SDS-PAGE was performed as described by Lanford and Butel (1979). The stacking gel was 5% acrylamide using a 30:0.8 acrylamide-to-bisacrylamide ratio. The separating gel was 10%, using a 100:1 acrylamide-to-bisacrylamide ratio.

Electrophoretic transfer of proteins from gels to nitrocellulose. Proteins were electrophoretically transferred from SDS gels to nitrocellulose filters and detected by antibody and ^{125}I-protein A as previously described (Slagle *et al.*, 1984).

Iodination of (C3H)MMTV by Enzymobead method. Detergent-solubilized (C3H)-MMTV was iodinated using Enzymobeads (Bio-Rad Laboratories, Richmond, Calif.), an immobilized preparation of lactoperoxidase and glucose oxidase, utilizing the procedure described by Soule *et al.* (1982). (C3H)MMTV (100 μg), 25 μl Enzymobeads, 1 mCi ^{125}I-Na (>500 mCi/ml, Amersham), and 50 μl 1% β-D-glucose were added to a small test tube, and the reaction mixture was incubated for 10 min at room temperature. The reaction was then quenched by running the mixture over a bovine serum albumin-pretreated PD10 column (Pharmacia, Piscataway, N. J.). Fifteen-drop fractions were collected, and the iodinated proteins were detected in the void volume.

Endoglycosidase H digestion. Primary cultures of BALB/cV tumor cells were iodinated *in situ* (as monolayers), extracted, and immunoprecipitated as described above. Immunoprecipitates were washed twice in 0.1 *M* sodium citrate buffer, pH 5.5, and the final pellets resuspended in 1.0 ml citrate buffer containing 100 μl Trasylol and either 15 μl TBS (control) or 15 μl (15 munit) endoglycosidase H (EndoH; Miles Laboratories, Inc., Elkhart, Ind.). Samples were incubated for 14 hr at 37° on a rotating platform,

were washed in TBS, and the pellets solubilized in gel sample buffer and analyzed by SDS-PAGE.

Metabolic labeling of cells. For metabolic labeling experiments, primary BALB/cV tumor cell cultures were starved in glucose-free media for 30 min (glucose-free Eagle's media containing 2% dialyzed FBS, 0.1 μg/ml gentamicin sulfate, 10 μg/ml insulin, 2 μg/ml dexamethasone, and 0.075% sodium bicarbonate). The glucose-free media were removed from cells and replaced with 1.0 ml per 100-mm plate of the same media containing either 200 μCi [^{3}H]glucosamine (25 Ci/mmol; ICN Pharmaceuticals, Inc., Irvine, Calif.) or 300 μCi L-[^{35}S]methionine ([^{35}S]Met; 1210 Ci/mmol; Amersham). At the end of a 3-hr labeling period at 37°, cells were rinsed and extracted (EB, 4°, 4 hr). Clarified extracts were analyzed for trichloroacetic acid (TCA) counts (see below).

External immunoprecipitation of cell surface MMTV proteins. Confluent cell monolayers were first starved for 2 hr in methionine-deprived media (D-MEM containing 0.1X methionine, 2% dialyzed FBS, 0.1 μg/ml gentamicin sulfate, 10 μg/ml insulin, 2 μg/ml dexamethasone, and 0.3% sodium bicarbonate), and then labeled for 1 hr in 1.5 ml per 100-mm plate of the same media supplemented with 130 μCi/ml of L-[^{35}S]methionine (Amersham/Searle Corp.). Radiolabeled cell monolayers were then subjected to external antibody immunoprecipitation as described by Santos and Butel (1982). Briefly, cell monolayers were rinsed with cold TBS, placed on ice, and incubated with 1 ml media containing 50 μl heat-inactivated rabbit antiserum (30 min, 4°) as described in the legend to Fig. 7. Unattached antibody was removed by extensive rinsing with cold TBS, and cells were disrupted in EB. Immune complexes (representing surface-exposed antigens complexed with antibodies) were removed from clarified extracts by the addition of heat-inactivated, formalin-fixed *Staphylococcus aureus* Cowan strain I (SACI; Kessler, 1975) as described previously (Santos and Butel, 1982). Final immunoprecipitates were analyzed by SDS gels and autoradiography. For chase experiments, cells were incubated in media containing excess unlabeled methionine for variable time periods prior to the antibody adsorption step.

Tunicamycin inhibition. Tunicamycin (TM; Calbiochem-Behring Corp.) was resuspended to 100 μg/ml in distilled water, pH 8.0. Primary cell cultures were then incubated for 19 hr in media containing 0, 0.5, 1.0, or 1.5 μg/ml TM, as previously described (Jarvis and Butel, 1985). Metabolic labeling (described above) was performed during the final 3 hr of the 19-hr incubation and was done in the presence of the appropriate TM concentration.

TCA precipitation. Forty microliters of labeled whole cell extracts (^{3}H or ^{35}S, described above) were spotted onto triplicate glass fiber filters (Whatman, No. 934-AH; Fisher Scientific Co., Pittsburgh, Pa.), and the filters dried (80°, 20 min). Proteins were then precipitated by incubating the filters sequentially in cold (4°) 10% TCA, 5% TCA, 5% TCA, and 95% ethanol for 5 min each. Filters were then dried (80°, 30 min), placed in Liquiscent (National Diagnostics, Somerville, N. J.), and the radioactivity was determined using a Beckman LS-250 liquid scintillation spectrometer.

External labeling of cell surface carbohydrate by tritiated sodium borohydride method. Primary cultures of BALB/cV tumor cells were labeled *in situ* with tritiated sodium borohydride [NaB^3H_4; >20 Ci/mmol; Amersham] using a modification of the procedure previously described (Gahmberg and Hakomori, 1973; Gahmberg, 1978). Briefly, monolayers were rinsed three times with TBS and a fourth time with D-PBS, pH 7.0. Two milliliters of TBS containing 50 units of heat-inactivated neuraminidase (Calbiochem-Behring Corp.) and 40 units of heat-inactivated galactose oxidase (Millipore Corp., Freehold, N. J.) were added per 75-cm^2 flask, and the culture was incubated for 30 min at 37°. Monolayers were then washed three times in D-PBS, and 2.0 ml of TBS containing 1 mCi NaB^3H_4 was added to each flask and incubated for 30 min at room temperature. Monolayers were then rinsed three times with cold TBS, extracted in

EB, immunoprecipitated, and analyzed by SDS-PAGE. Gels were then impregnated with Autofluor (National Diagnostics), dried, and exposed to X-ray film at −70°.

Electron microscopy. Random fragments of a BALB/cV tumor were removed and washed three times in phosphate-buffered saline (40 m*M* sodium phosphate, pH 7.2, 150 m*M* NaCl), fixed in 3% glutaraldehyde in 0.1 *M* PIPES buffer (Sigma; pH 7.4), and postfixed in 2% osmium tetroxide in 0.1 *M* PIPES. Tissues were then stained *en bloc* with 2% aqueous uranyl acetate, and embedded in Epon (EMS):Araldite (Polysciences). Samples were sectioned, stained with lead citrate, and examined in an RCA EMU3 transmission electron microscope at 100 kV.

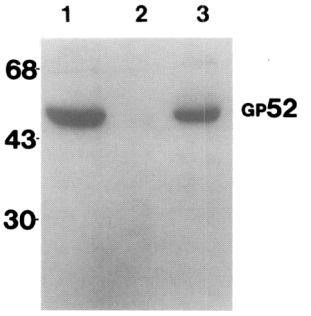

FIG. 1. MMTV-specific proteins detected on the surface of MMTV-producer and -nonproducer cell lines. Cell monolayers were iodinated, extracted, immunoprecipitated with anti-(C3H)MMTV, and the final immunoprecipitates analyzed by 14% SDS-PAGE and autoradiography. Cell lines analyzed included Mm5mt/cl (lane 1), MTV-L (lane 2), and H-1 (lane 3). Molecular-weight markers are indicated on the left.

RESULTS

Identification of MMTV Proteins Expressed on the Surface of (C3H)MMTV-Producing Cells and (BALB/cV)-MMTV-Positive Tumor Cells

Three different established mouse mammary tumor cell lines were examined for cell surface expression of MMTV antigens. Cells grown to near confluence were rinsed three times with TBS and intact monolayers were iodinated using the lactoperoxidase-catalyzed reaction described under Materials and Methods. After labeling, cell monolayers were rinsed with cold TBS, extracted, immunoprecipitated using rabbit anti-(C3H)MMTV[d], and the immunoprecipitates were analyzed on 14% SDS gels.

Mm5mt/cl and H-1 cells, both lines which produce (C3H)MMTV, were found to express MMTV gp52 at the cell surface (Fig. 1, lanes 1 and 3). (The faint band visible at 68K in lanes 1 and 3 was not obtained in repeated experiments.) MTV-L cells, which do not produce virus particles, were shown to lack detectable amounts of gp52 on the cell surface (Fig. 1, lane 2). No iodinated proteins were detected when normal rabbit serum was used in the immunoprecipitation (data not shown). The surface specificity of the iodination reaction was demonstrated by the fact that no other MMTV structural proteins were detected. These results confirm previous observations that MMTV gp52 is expressed on the surface of MMTV-positive cells (Yang *et al.*, 1977; Schochetman *et al.*, 1978; Massey and Schochetman, 1979).

Primary cultures of a BALB/cV tumor were analyzed for cell surface expression of viral proteins using the same iodination procedure. In contrast to the MMTV-producing cell lines, BALB/cV cells lacked detectable gp52 at the cell surface. Instead, a 68K protein was identified (Fig. 2, lane 2). Monospecific antisera prepared against MMTV gp52/gp36 (Fig. 2, lane 3) or p28 (Fig. 2, lane 4) identified the 68K protein as being *env* related (Fig. 2, lane 3).

Several organs from adult BALB/cV mice were analyzed for the presence of $68K^{env}$ (Table 1). $68K^{env}$ was present in lactating mammary gland (LMG), preneoplastic Cv-2 and Cv-4 mammary tissue, BALB/cV tumor tissue, and MTV-L cells. All other organs, including the mammary gland from a virgin BALB/cV mouse (virgin mammary gland, VMG), were negative for the expression of $68K^{env}$, as well as for correctly processed MMTV proteins, gp52 and p28 (Table 1). Thus, the expression of $68K^{env}$ appears to be restricted to mammary tissues that also express other MMTV-specified proteins.

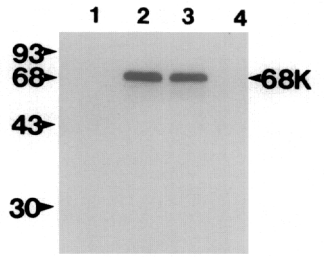

FIG. 2. MMTV-specific proteins expressed on the surface of primary cultures of BALB/cV tumor cells. Intact cell monolayers were iodinated, extracted, immunoprecipitated, and the immunoprecipitates analyzed by 14% SDS-PAGE and autoradiography. Sera used for immunoprecipitation included normal rabbit serum (lane 1), anti-(C3H)MMTV (lane 2), anti-gp52/gp36 (lane 3), and anti-p28 (lane 4). Molecular-weight markers are indicated on the left.

Biochemical Characterization of Surface 68K^{env}

The cell surface localization of the 68K^{env} protein, in the absence of mature gp52, was unexpected. Therefore, the protein was extensively characterized to understand its aberrant cell surface expression. It is known that the MMTV *env* precursor, as well as gp52 and gp36, are modified by glycosylation (Anderson *et al.*, 1979; Dickson and Atterwill, 1980). We used three different approaches to investigate whether the surface 68K^{env} was undergoing biochemical modifications similar to those reported for the MMTV *env* precursor in other systems.

The first approach used in characterizing the surface 68K^{env} protein involved the use of EndoH, a glycosidic enzyme known to cleave at the site of attachment of asparagine-linked glucosamine to the core oligosaccharide (Tarentino and Maley, 1974; Tarentino *et al.*, 1974). Since it has previously been shown that the *env* precursor is modified by N-linked, high-mannose glycosylation, it was predicted that a similarly modified 68K^{env} protein would be sensitive to EndoH digestion. Primary tumor cells were iodinated, extracted, immunoprecipitated, and the immunoprecipitates subjected to EndoH digestion as described under Materials and Methods. The mobility of BALB/cV surface 68K^{env} in SDS gels was not affected by incubation with EndoH (Fig. 3, lane 4). As a control for enzyme activity, ^{125}I-labeled (C3H)-MMTV was immunoprecipitated and digested under identical conditions. gp52, which is modified by N-linked, high-mannose glycosylation (Dickson and Atterwill, 1980; Jarvis and Butel, 1985), showed a decrease in molecular weight upon treatment with EndoH (Fig. 3, lane 7; see arrow), consistent with the loss of carbohydrate. The migration of gp36, an N-linked, complex-type glycoprotein (Dickson and Atterwill, 1980), was not affected

TABLE 1

EXPRESSION OF MMTV PROTEINS IN TISSUES OF ADULT BALB/cV MICE

Sample tested[a]	Presence of MMTV proteins by immunoblot analysis[b]		
	p28	gp52	68K^{env}
VMG	–	–	–
LMG	+[c]	+[c]	+
Heart	–	–	–
Liver	–	–	–
Kidney	–	–	–
Pancreas	–	–	–
Spleen	–	–	–
Cv-2 HAN	+	+	+
Cv-4 HAN	ND[d]	ND	+
Cv-2 tumor	+	+	+
MTV-L cells[e]	–	–	+

[a] Tissues and cells were extracted as described previously (Slagle *et al.*, 1984), and an aliquot was separated by SDS-PAGE.

[b] Separated proteins were then transferred to nitrocellulose (600 mA, overnight, 4°) and probed using anti-(C3H)MMTV, anti-gp52/gp36, and ^{125}I-protein A. A positive result indicates that the protein band was visible on the autoradiogram. A negative result indicates that no protein band was visible on the autoradiogram following prolonged exposure of film (sensitivity of detection, 5 ng; unpublished observation).

[c] One of seven BALB/cV LMG extracts contained 68K^{env} only and lacked detectable levels of p28 and gp52.

[d] ND = not done.

[e] The MTV-L cell line was established from a virus-positive BALB/cV mammary tumor (Butel *et al.*, 1977).

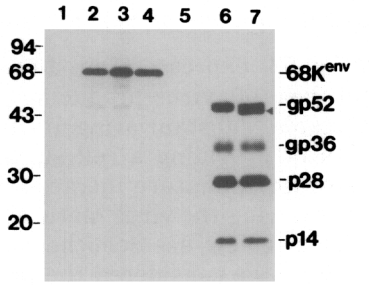

FIG. 3. Effect of EndoH on (BALB/cV)MMTV surface 68K^{env}. BALB/cV tumor cell cultures were iodinated, extracted, and immunoprecipitated (lanes 1–4). Disrupted (C3H)MMTV was iodinated and immunoprecipitated (lanes 5–7). Final immunoprecipitates were incubated for 14 hr in the presence of 15 μl (15 munit) EndoH (lanes 4 and 7) or 15 μl buffer only (lanes 1–3, 5, and 6). Immunoprecipitates were then washed and analyzed by 14% SDS-PAGE and autoradiography. Sera used for immunoprecipitation included normal rabbit serum (lanes 1 and 5), anti-(C3H)MMTV (lanes 2, 4, 6, and 7), and anti-gp52/gp36 (lane 3). Molecular-weight markers are indicated on the left. Arrow (lane 7) denotes faster migrating gp52 of (C3H)MMTV following EndoH treatment.

by EndoH treatment (Fig. 3, lane 7). The glycosidic specificity of the enzyme was demonstrated by the fact that nonglycosylated p28 and p14 were not affected by EndoH digestion (Fig. 3, lane 7). These results indicate that surface 68K^{env} is not modified by N-linked, high-mannose-type glycosylation.

It has been demonstrated that, although most of the MMTV *env*-precursor proteins are processed as high-mannose glycoproteins that are subsequently cleaved into gp52 and gp36, a small population of the precursor polyprotein is converted to a complex oligosaccharide by the addition of fucose and galactose (Dickson and Atterwill, 1980; Sarkar and Racevskis, 1983). Complex oligosaccharides, which are EndoH resistant, are sensitive to the inhibitor of glycosylation, tunicamycin (TM), that inhibits the *en bloc* transfer of preassembled oligosaccharides from a lipid carrier to the newly synthesized protein (Leavitt *et al.*, 1977). It was possible that the BALB/cV 68K^{env} surface protein may have been modified in that way. Therefore, MMTV proteins expressed at the cell surface in the presence of TM were identified. Primary cultures of BALB/cV tumor cells were grown for 19 hr in the presence of TM. During the final 3 hr of incubation, cells were starved in glucose-free media (30 min) and metabolically labeled with either [^{3}H]glucosamine or [^{35}S]Met, as described under Materials and Methods. At the end of the labeling period, half of the duplicate plates were extracted and processed for TCA-precipitable counts. Cells in the remaining duplicate plates were iodinated, extracted, immunoprecipitated, and the immunoprecipitates analyzed on SDS gels.

Cells grown in the presence of 1.5 μg/ml TM showed a 50% decrease in [^{3}H]glucosamine incorporation (as compared to control, untreated cells), while [^{35}S]Met incorporation into TCA-precipitable counts was unaffected at this concentration of TM (Fig. 4B). The cell surface expression of 68K^{env} was monitored in the TM-inhibited cells (Fig. 4A), and no decrease in the molecular weight of 68K^{env} was noted (Fig. 4A, lane 4B). The amount of surface 68K^{env} present did not appear to decrease in the presence of TM, although this procedure did not allow precise quantitation of 68K^{env} synthesis. These data are consistent with the EndoH results and suggest that surface 68K^{env} is not modified by the addition of N-linked, complex-type oligosaccharides.

Dickson and Atterwill (1980) have demonstrated that the subpopulation of the MMTV *env* precursor that is expressed at the cell surface contains galactose. That protein can, therefore, be detected by a cell surface labeling procedure that involves treating cells with galactose oxidase, followed by a reduction in the presence of [^{3}H]sodium borohydride. This method was employed in a final effort to determine if surface 68K^{env} was glycosylated. Primary cultures of BALB/cV tumor cells were labeled as described under Materials and Methods, extracted, immunoprecipitated, and the immunoprecipitates analyzed by SDS-PAGE, fluorography, and autoradiography. As a control, a serially transplanted tumor known to express both gp52 and 68K^{env} on the

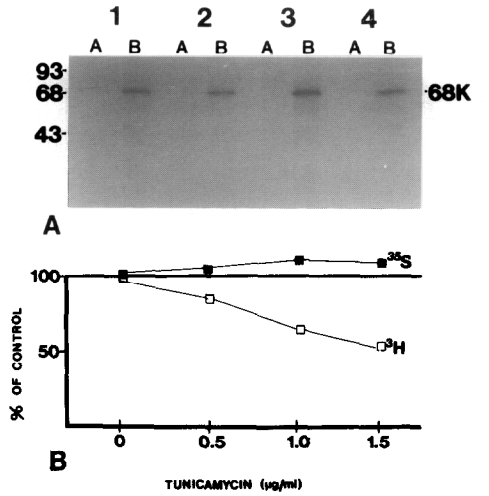

FIG. 4. Effect of tunicamycin on BALB/cV surface 68K^{env}. Near-confluent BALB/cV tumor cells were grown in the presence of TM for 19 hr. (A) Monolayers were then iodinated, extracted, immunoprecipitated, and the immunoprecipitates analyzed by 12% SDS-PAGE and autoradiography. Concentrations of TM used were 0 (lane 1), 0.5 (lane 2), 1.0 (lane 3), and 1.5 (lane 4) µg/ml. Sera used for immunoprecipitation included normal rabbit serum (lanes 1A–4A) and anti-(C3H)MMTV (lanes 1B–4B). Molecular-weight markers are indicated on the left. (B) Duplicate cell cultures were treated as above and, during the final 3 hr of the 19-hr TM incubation, were metabolically labeled with both [^{3}H]glucosamine and [^{35}S]Met. Cells were then extracted, and clarified extracts were analyzed for TCA-precipitable counts. ^{3}H and ^{35}S cpm obtained from cells grown in the presence of TM were compared to those obtained from control (non-TM-treated) cells.

cell surface (Slagle *et al.*, 1981; Fig. 5, lane 2) was subjected to this labeling procedure in parallel. We were able to identify galactose-containing gp52 (Fig. 5, lane 5), but not 68K^{env} on the surface of these control cells. We were unable to identify either 68K^{env} or gp52 on the surface of BALB/cV primary tumor cells using this procedure (data not shown). The specificity of the oxidation-reduction reaction was demonstrated by the fact that galactose oxidase was required for the labeling of gp52 (Fig. 5, lane 4). These data provide additional evidence that the BALB/cV surface 68K^{env} is not modified by glycosylation.

Biological Function of Surface 68K^{env}

The gp52 expressed on the plasma membranes of virus-producing cells is quite stable, substantiating its proposed function of providing a cell surface budding site for immature intracellular core particles during the virus maturation process (for a review, see Schochetman *et al.*, 1980). Since we considered the possibility that BALB/cV surface 68K^{env} might provide a similar function, the stability of 68K^{env} in the plasma membrane was determined. Primary BALB/cV tumor cells were grown as monolayer cultures and iodinated. At the end of the labeling period, some cultures were extracted immediately while companion cultures were rinsed, fresh serum-free media added, and the cells reincubated for variable chase periods before extraction and immunoprecipitation.

The gp52 present on the cell surface of control Mm5mt/c1 cells was found to be stable during a 30-min chase period (Fig. 6). Longer chase periods established that gp52 was stable on these cells for at least 2 hr (data not shown). In contrast, the

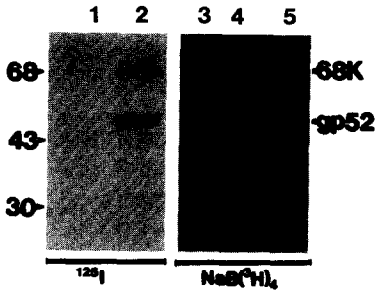

FIG. 5. NaB^3H_4 labeling of cell surface carbohydrates. Primary cell cultures of a control serially transplanted BALB/cV tumor previously shown to express both surface 68K^{env} and gp52 (Slagle *et al.*, 1981) were iodinated or labeled by NaB^3H_4. Cells were then extracted, immunoprecipitated, and the immunoprecipitates analyzed by 14% SDS-PAGE and autoradiography or fluorography. Sera used for immunoprecipitation included normal rabbit serum (lanes 1 and 3) and anti-(C3H)MMTV (lanes 2, 4, and 5). Molecular-weight markers are indicated on the left. MMTV gp52 was not labeled in the absence of galactose oxidase (lane 4).

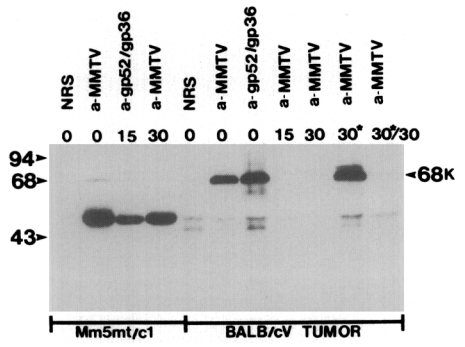

FIG. 6. Stability of MMTV proteins present at the surface of Mm5mt/cl and BALB/cV tumor cells. Following iodination of cell monolayers, cells were extracted either immediately (0 min) or after incubation (15 or 30 min) in serum-free media. Cell extracts were clarified, the MMTV-reactive polypeptides immunoprecipitated using the sera listed at the top of each lane, and the immunoprecipitates analyzed by 14% SDS-PAGE and autoradiography. Molecular-weight markers are shown at the left. Newly inserted 68K^{env} could be labeled on the surface of BALB/cV tumor cells which had previously been iodinated and chased for 30 min (see lane 30*). This newly inserted 68K^{env} was rapidly shed during subsequent chase (lane 30*/30).

68K^{env} protein present on BALB/cV tumor cells was rapidly lost from the cell surface and was completely absent after only a 15-min chase (Fig. 6). Newly synthesized 68K^{env} was rapidly reinserted into the plasma membrane and could be iodinated on cells that had been previously iodinated and then chased for 30 min (Fig. 6, see asterisk).

A different experimental approach was used to address the possibility that the instability of 68K^{env} might be induced by the iodination process per se, rather than being an intrinsic property of the protein. Primary cultures of BALB/cV tumor cells were starved for 2 hr in methionine-free media and were then metabolically labeled for 1 hr with [^{35}S]Met. Intact cell monolayers were rinsed with cold TBS, placed on ice, and reacted with specific antisera to detect ^{35}S-labeled MMTV proteins expressed on the cell surface. Excess antibody was rinsed away, the cells were extracted, and SACI was added to remove immune complexes from the clarified extracts. Final immunoprecipitates were analyzed by SDS-PAGE and autoradiography.

Three high-molecular-weight MMTV-specific proteins were detected by the external antibody technique: 79K^{gag} (Fig. 7, lane 3), 77K^{gag} (Fig. 7, lane 3), and 68K^{env} (Fig. 7, lanes 3 and 4). The *gag* precursors detected by this procedure, which were not accessible for cell surface iodination (Fig. 2), were probably precipitated as part of budding virus at the cell surface. The stability of these three proteins within the plasma membrane was demonstrated by chasing the pulse-labeled cells in unlabeled media prior to the antibody reaction. Whereas the 77K^{gag} was stable during the chase periods examined (Fig. 7, lanes 5-7), both the 79K^{gag} and the 68K^{env} were turned over rapidly and were almost completely absent following a 45-min chase (Fig. 7, lanes 5-7). The longer half-life of 68K^{env} at the cell surface in this experiment, as compared to iodinated 68K^{env} (Fig. 6), is possibly explained by the additional time needed for ^{35}S-labeled intracellular 68K^{env} to move to the cell surface. These data, based on metabolic labeling

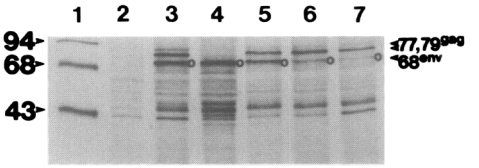

FIG. 7. Detection of MMTV cell surface proteins by external antibody immunoprecipitation. Following metabolic labeling of BALB/cV cells, monolayers were either immediately rinsed in cold TBS and placed at 4° (lanes 2, 3, and 4) or chased in methionine-containing media for 15 (lane 5), 30 (lane 6), or 45 (lane 7) min prior to being rinsed and placed at 4°. Radiolabeled monolayers were then incubated with either NRS (lane 2), anti-gp52/gp36 (lane 4), or anti-(C3H)MMTV (lanes 3, 5, 6, and 7) for 30 min at 4°. Nonbound antibody was removed by rinsing, and the cells were extracted and processed as described under Materials and Methods. ^{14}C-labeled molecular-weight markers are shown in lane 1. Note that both 79K^{gag} and 68K^{env} are rapidly turned over at the cell surface. In contrast, 77K^{gag} remains stable during the chase periods examined.

coupled with external immunoprecipitation, indicate that the 68K^{env} synthesized by BALB/cV tumor cells is rapidly turned over at the cell surface, and confirm results obtained by the iodination procedure (Fig. 6).

The fate of the ^{125}I-labeled 68K^{env} released from the cells was investigated by centrifugations of media collected at the end of a 30-min chase period. Media containing ^{125}I-labeled 68K^{env} were clarified (15,000 rpm, 30 min), followed by centrifugation at 100,000 *g* (1 hr) onto a 30% sucrose cushion. ^{125}I-labeled 68K^{env} was immunoprecipitated from the supernatant of the high-speed centrifugation (Fig. 8, lanes 2 and 3), an observation compatible with this protein existing in soluble form. ^{125}I-labeled 68K^{env} also was present in the 100,000 *g* pellet. The presence of other viral proteins in this pellet (data not shown) provides indirect evidence that some of the surface 68K^{env} may be incorporated into virus particles. However, numerous attempts to localize shed ^{125}I-labeled 68K^{env} into material banding at a density of 1.16–1.18 g/cc on a sucrose gradient have been unsuccessful.

Since the exclusive localization of the MMTV *env* precursor at the cell surface is usually associated with a block in virus

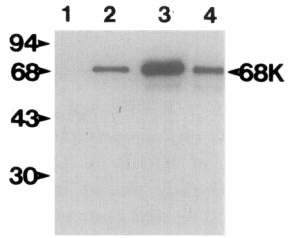

FIG. 8. Recovery of ^{125}I-labeled 68K^{env} released into the media. BALB/cV tumor cells were iodinated, and the media from a 30-min chase were collected and clarified (15,000 rpm, 30 min). ^{125}I-labeled 68K^{env} was then immunoprecipitated from the supernatant (lanes 1–3) and the pellet (lane 4) of a high-speed centrifugation (100,000 *g*, 1 hr, onto a 30% sucrose cushion). Sera used for immunoprecipitation included normal rabbit serum (lane 1), anti-(C3H)MMTV (lanes 2 and 4), and anti-gp52/gp36 (lane 3). Molecular-weight markers are indicated at the left.

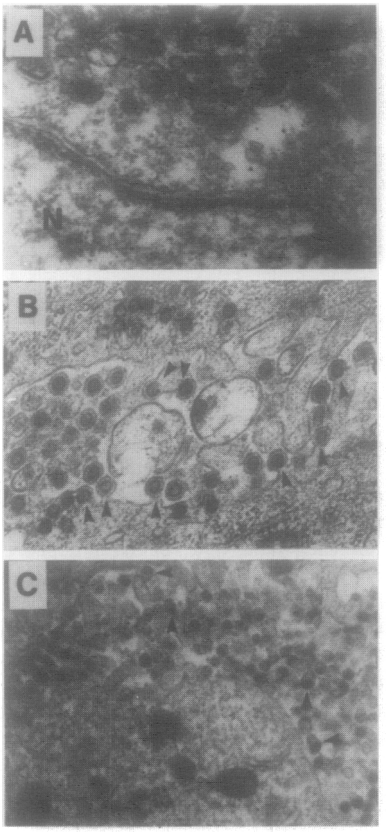

FIG. 9. Virus production by BALB/cV mammary tumor cells. A primary BALB/cV tumor was processed for electron microscopy and examined for the presence of virus particles. (A) Numerous intracytoplasmic type A particles are seen (N = nucleus). 28,800X. (B) Virus particles are observed budding into intercellular spaces (see arrows). 28,800X. Extracellular viruses with type-B morphology (see arrows) are noted in (C). 19,200X.

maturation (Nusse *et al.*, 1979; Racevskis and Sarkar, 1982; Slagle *et al.*, 1985), we next determined if BALB/cV tumor cells were producing mature B-type MMTV particles. Random segments of a BALB/cV primary tumor were fixed, sectioned, and examined by electron microscopy. The remainder of the tumor was established as a primary cell culture, iodinated, and shown to express surface 68K^{env} (data not shown). Electron micrographs revealed numerous intracytoplasmic A-type particles (Fig. 9A), as well as virus particles budding into intercellular spaces (Fig. 9B,

see arrows). Type-B morphology, typical of MMTV, was noted with the extracellular virus particles (Fig. 9C, see arrows).

DISCUSSION

This report describes a thorough analysis of the expression of MMTV-specific proteins on the surface of BALB/cV mammary tumor cells. In contrast to other MMTV-producing systems in which gp52 is the main viral cell surface protein detected (for a review, see Schochetman *et al.*, 1980), the BALB/cV tumor cells lack detectable levels of MMTV gp52 on the cell surface. Instead, we identified a 68K *env*-related protein. The finding of a high-molecular-weight form of the MMTV *env* protein on the cell surface in the absence of properly processed gp52 is not unique to the BALB/cV system, having been reported for GR lymphoma cells (Nusse *et al.*, 1979), DBA/2 leukemia cells (Racevskis and Sarkar, 1982), and BALB/c D-2 preneoplastic mammary cells (Slagle *et al.*, 1985). In those reports, the aberrant expression of an unprocessed *env* precursor at the cell surface was associated with a lack of virus production. Therefore, the BALB/cV system differs from those in that type B virus particles are readily detected by electron microscopy in BALB/cV tumors (see Fig. 8).

The normal maturation pathway for the MMTV *env* gene has been well defined. The 24 S *env*-specific mRNA is translated on membrane-bound ribosomes (Dickson and Atterwill, 1980), resulting in a 66K-68K polyprotein (Robertson and Varmus, 1979; Dudley and Varmus, 1981; Arthur *et al.*, 1982) from which a leader sequence is cotranslationally removed (Dickson *et al.*, 1982; Arthur *et al.*, 1982). The 60K apoprotein (Dickson and Atterwill, 1980; Arthur *et al.*, 1982) is cotranslationally modified by glycosylation, resulting in the mature *env* precursor, designated $Pr70^{env}$ (Sarkar and Racevskis, 1983) or $Pr73^{env}$ (Dickson and Atterwill, 1980; Robertson, 1984). Recent studies have demonstrated the existence of at least two populations of $Pr70^{env}$. The majority of $Pr70^{env}$ is cleaved into gp52 and gp36 *en route* to the cell surface; once at the cell surface, only gp52 is accessible to iodination (Yang *et al.*, 1977; Schochetman *et al.*, 1978; Massey and Schochetman, 1979). A second population of $Pr70^{env}$ is not cleaved into gp52 and gp36, but instead is modified further by complex-type glycosylation (Anderson *et al.*, 1979; Dickson and Atterwill, 1980; Racevskis and Sarkar, 1982; Sarkar and Racevskis, 1983). This population, now designated $Pr75^{env}$ (Sarkar and Racevskis, 1983) or $Pr73^{env}$ (Dickson and Atterwill, 1980), can be detected at the cell surface (Dickson and Atterwill, 1980) as well as in the media (Sarkar and Racevskis, 1983) of MMTV-producing cells.

Although the precise nature of the surface $68K^{env}$ processing defect noted in this study is unknown, the size of the protein is compatible with at least four possibilities, based on the above information. First, the $68K^{env}$ protein may represent an MMTV *env* precursor from which the leader sequence has not been removed. The size of the predicted MMTV leader sequence varies (11,000, 7000, or 5700 Da), depending on which of the three potential methionine starts is utilized *in vivo* (Redmond and Dickson, 1983; Majors and Varmus, 1983). Thus, the BALB/cV $68K^{env}$ is approximately the size expected of a 60K apoprotein plus an uncleaved 7000-Da leader. The molecular process that might allow a protein to retain its leader sequence is unclear. One possible explanation involves the intracellular location of *env* mRNA translation. In the avian sarcoma virus system, 10% of the $pp60^{src}$-specific mRNA is translated on membrane-bound ribosomes (presumably resulting in plasma membrane-localized $pp60^{src}$), while the remaining 90% is translated on free ribosomes (resulting in cytoplasmic localization of the protein; Purchio *et al.*, 1980). Any MMTV *env* mRNA similarly translated on free ribosomes might be expected to retain its leader sequence. However, the mechanism by which the $68K^{env}$ would then get transported to the cell surface is unknown.

A second explanation for the processing defect of 68K^{env} is based on the observation that the *env* gene of the endogenous Mtv-8 provirus of GR mice has been shown to be defective. A mutation giving rise to a stop codon results in a truncated 68K^{env} precursor which is not processed into gp52 and gp36 (Groner *et al.*, 1984; G. Knedlitschek and N. Kennedy, personal communication). Such a truncated *env* protein would lack the hydrophobic "membrane anchor" region of gp36 (Redmond and Dickson, 1983; Majors and Varmus, 1983). It remains to be determined whether the *env* gene of Mtv-8 in BALB/c mice contains the same termination codon as observed in Mtv-8 of GR mice. Such a mutation conceivably could result in the phenomena of aberrant processing and instability of the protein in the plasma membrane reported for 68K^{env} in this paper. A similarly truncated *env* precursor in the BALB/cV system would have to retain an 11K leader sequence to achieve the observed 68K size.

The third possibility for the origin of the 68K^{env} processing defect is that 68K^{env} is the normal, glycosylated *env* precursor, which does not get cleaved into gp52 and gp36 and is inappropriately expressed at the cell surface. However, the data presented in this paper are not consistent with this possibility. 68K^{env} was shown to be resistant to both EndoH (Fig. 3) and TM (Fig. 4), suggesting that 68K^{env} is not modified by N-linked glycosylation. We are unable to rule out the possibility that 68K^{env} may be modified by the less well understood O-type glycosylation, which has been reported for a glycoprotein of coronaviruses (Holmes *et al.*, 1981; Niemann and Klenk, 1981), as well as for SV40 tumor (T) antigen (Jarvis and Butel, 1985). O-linked glycosylation, which is TM and EndoH resistant, has not been reported for a glycoprotein of MMTV.

Finally, it is possible that 68K^{env} represents a fusion protein consisting of some *env* sequences and those from another gene, either viral or cellular in origin. Since we used *env*-specific antisera to characterize 68K^{env} rather than individual antisera monospecific for gp52 and gp36, we have not demonstrated unequivocally that 68K^{env} is indeed the *bona fide* MMTV *env* precursor. Such a phenomenon, resulting in the generation of a fusion protein, has not been described for the MMTV system.

The inability to detect gp52 on the surface of BALB/cV tumor cells is unexpected in view of the fact that intracellular gp52 and gp36 can be identified (Slagle *et al.*, 1984) and virus particles can be seen budding from the cell surface (see Fig. 9). Several possible explanations for this observation can be considered. Mature gp52 may indeed be in the plasma membrane, but oriented such that it is inaccessible not only to surface iodination (Figs. 2–4, 6) but also to labeling by the NaB^3H_4 technique. Alternatively, gp52 may be present in its normal conformation, but may be interacting with 68K^{env} such that it is hidden by the larger protein and unavailable for labeling. Such an interaction would involve a gp36 portion of 68K^{env}, since gp52 and gp36 have been shown to associate in forming the spikes of the viral envelope (Dion *et al.*, 1979; Westenbrink and Koornstra, 1979; Racevskis and Sarkar, 1980). It is also possible that properly oriented surface gp52 is present, but at levels below detection using the available techniques. Finally, we must consider the possibility that BALB/cV tumor cells lack cell surface gp52 and that 68K^{env} is providing the function of serving as the cell surface budding site for maturing virus particles (discussed below).

The cell surface expression of 68K^{env}, in the absence of detectable surface gp52, appears to be a defect in the provirus, rather than in the ability of the cells to correctly process the *env* precursor. The latter phenomenon has been described for the *env* gene of AKR virus-infected rat cells (van der Hoorn *et al.*, 1983) and the *gag* and *env* genes of MuLV-infected rat cells (Fitting *et al.*, 1981). In BALB/cV tumor cells, however, the intracellular glycosylated forms of Pr70^{env}, gp52, and gp36 are present (Slagle *et al.*, 1984; unpublished observations), suggesting that

the cells contain the enzymes necessary to correctly process a normal MMTV *env* gene.

Since BALB/cV tumors contain several MMTV proviruses (Drohan *et al.*, 1981), it is difficult to determine which provirus is serving as the template for 68K^{env} expression. We must consider the possibility that the MMTV expression observed in BALB/cV tumors is coming from more than one proviral template. For example, the 68K^{env} might be expressed from a defective provirus, while the virus particles are produced from the proviral template of the milk-transmitted (BALB/cV)MMTV. Alternatively, the milk-transmitted proviral template might also be defective. In this scenario, the properly processed *env*-gene products found in BALB/cV tumor cells could be explained by occasional readthrough of a termination codon in the *env* gene of Mtv-8. A final consideration in determining the template for 68K^{env} expression is that the primary tumors in this study arose from a dimethylbenzanthracene (DMBA)-induced preneoplastic HAN outgrowth line (Cv-2; Slagle *et al.*, 1984). The aberrant processing of the BALB/cV *env* gene is not unique to this particular outgrowth line, nor is it due to a mutagenic effect of DMBA treatment, because the same surface 68K^{env} can be detected on normal mammary tissue from lactating BALB/cV mice and in hormone-induced preneoplastic BALB/cV tissue (Table 1).

The biologic role, if any, of surface 68K^{env} is unknown, although several interesting possibilities can be envisioned. First, 68K^{env} might be involved in virus maturation. The incorporation of viral precursor proteins into rapid-harvest virus has been reported for other oncornaviruses (Jamjoon *et al.*, 1975; Oskarsson *et al.*, 1975; Shapiro and August, 1976). However, the marked instability of 68K^{env} in the plasma membrane, as compared to the stability of cell surface gp52 of Mm5mt/c1 cells (see Figs. 6, 7), suggests that 68K^{env} is not involved in virus maturation. The recovery of some shed 68K^{env} in a 100,000 *g* pellet provides circumstantial evidence that 68K^{env} might be virus associated. Although we have been unable to definitively demonstrate the presence of 68K^{env} in the BALB/c virus particle, we cannot rule out the possibility that some 68K^{env} is occasionally incorporated into virus.

A second putative function for surface 68K^{env} centers on the fact that much of the ^{125}I-labeled 68K^{env} shed from cultured BALB/cV tumor cells can be recovered as a soluble protein (see Fig. 8). This shed 68K^{env} is stable and is not converted to a lower molecular-weight form during the several hours of chase period examined (data not shown). The shedding of proteins from the surface of tumor cells has been proposed as a mechanism by which growing tumor cells escape elimination by the host immune system (Alexander, 1974; Nordquist *et al.*, 1977; Grossman and Berke, 1980; Van Blitterswijk *et al.*, 1975). It is conceivable that shed 68K^{env} might provide just such a biologic function in BALB/cV mice. MMTV antigens have been identified in the serum of tumor-bearing mice (Hilgers *et al.*, 1973; Verstraeten *et al.*, 1975; Ritzi *et al.*, 1976; Zangerle *et al.*, 1977; Schochetman *et al.*, 1979). However, since antibody to MMTV is not protective against tumors (Muller *et al.*, 1971; Ihle *et al.*, 1976; Miller *et al.*, 1977; Arthur *et al.*, 1978) and since MMTV antigens can be detected concurrently with MMTV antibodies in the sera of tumor-bearing mice (Arthur *et al.*, 1978), the possibility that shed viral proteins serve as blocking factors in modulating the host immune response to growing mammary tumors remains intriguing.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the help and advice of Daniel Medina. We also thank Ed Calomeni for the electron micrographs in this report. This study was supported in part by Research Grants CA 25215 and CA 33369 from the National Cancer Institute, by National Service Research Award CA 09197 from the National Institutes of Health, and by an American Association of University Women Predoctoral Fellowship (awarded to B.L.S.).

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