

Cell Anchorage Determines Whether Mammary Tumor Virus Glycoproteins Are Processed for Plasma Membranes or Secretion

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ABSTRACT The subcellular localization of mouse mammary tumor virus (MMTV) glycoproteins was analyzed in infected and cloned rat hepatocarcinoma cells cultured with the MMTV transcriptional inducer dexamethasone. When reacted with protein A-coated erythrocytes in the presence of antisera specific for viral glycoproteins or with fluorescent antisera, only some of the cells acquired surface label. This diversity was dependent on cell anchorage to the substratum. In general, the more rounded, less adherent cells contained the MMTV glycoproteins on their surfaces, whereas the flatter, more adherent cells did not. After a change in adherence, a delay preceded complete remodeling of the plasma membranes. Fluorescent antibody studies of fixed cells and analyses of viral glycoprotein synthesis and shedding using L-[³⁵S]methionine indicated that the different expression of MMTV glycoproteins in round versus flat cells is caused by a switch in posttranslational processing. In round cells, the MMTV-encoded precursor glycoprotein is proteolytically cleaved and then transported to plasma membranes as a complex of two subunits, the smaller being the membrane anchor. In flat adherent cells, the smaller subunit is rapidly degraded in an intracellular organelle and the larger is then secreted into the medium. As indicated by labeling of cells with ¹²⁵I, the concentrations of several host-encoded plasma membrane components are also influenced by cell anchorage. We propose that this switch in cell surfaces and in secretions dependent upon cell-substratum attachments may be a common control mechanism important for embryogenesis, wound healing, and cancer.

Anchorage of cells can have dramatic effects on their plasma membrane and secretory protein compositions and (possibly as a consequence) on their abilities to respond to factors that control mitosis or differentiation (e.g., see references 1–4, 8, 10, 16, 22, 24, and 26). For example, corneal epithelial cells respond to fibroblast growth factor but not to epidermal growth factor when cultured on plastic, whereas the converse occurs on a strongly adherent extracellular matrix (10). Similarly, cancer cells are generally weakly adherent and they have abnormal membranes and growth factor responsiveness. Recent evidence has demonstrated that growth of cancer cells on highly adhesive substrata can alter their plasma membrane compositions and coordinately reduce their metastatic capabilities (16). “Density dependent” growth control of cultured fibroblasts may also be primarily caused by a reduction in

anchorage and a corresponding change in growth factor responsiveness that results from crowding or from a lowering of serum concentrations (8). Cell differentiation and wound healing may also be initiated by coupled changes in cell anchorage and cell surfaces (e.g., see references 1, 2, 4, 8, 13, 22, 25, 26).

Despite the importance and possible interrelatedness of these observations, little is known of the mechanisms by which plasma membrane and secretory protein compositions can be modulated by cell attachments. Recently, we initiated a study of mouse mammary tumor virus (MMTV)¹ glycoprotein synthesis in cultured rat hepatoma cells. MMTV transcription in

¹ Abbreviation used in this paper: MMTV, mouse mammary tumor virus.

these cells is induced above a basal level by dexamethasone (6, 7, 17). Moreover, it has been reported that the *env* gene of MMTV encodes a precursor glycoprotein that is processed in a complex manner to form a plethora of membranous and secretory products (6, 7, 20) and that the membranous products are synthesized only in the presence of dexamethasone (6). Using freshly cloned cells cultured with dexamethasone, we observed that the glycoprotein precursor is processed along different pathways in different cells and that the pathway switch is closely correlated with changes in cell anchorage. Moreover, the dexamethasone-dependent processing pathway (6) cannot occur in cells tightly anchored to their substrata.

MATERIALS AND METHODS

Cells and Viruses: The rat hepatoma cell line HTC (subclone B2.1) and its derivative, which had been infected at a high multiplicity with MMTV before subcloning (line M1.54), were generously provided by Gary Firestone and Keith Yamamoto (University of California, San Francisco) and grown as described (6, 7). Subclones were prepared for this study by plating at a limiting dilution into wells of a microtiter plate and using wells that contained one colony. Cells were maintained in either the presence or absence of $1 \mu\text{M}$ dexamethasone for at least 3 d before and during all experiments.

Antisera: Goat antisera to the MMTV glycoproteins gp52 (anti-gp52) and gp-36 (anti-gp36) and a normal goat serum were provided by the Virus Cancer Program, National Cancer Institute, Bethesda, MD. A rabbit antiserum to the MMTV *env* glycoproteins (anti-*env*) and the preimmune serum were generous gifts of Gary Firestone and Keith Yamamoto. A typical analysis of the specificity of these sera is shown in Fig. 1. The anti-gp36 and anti-*env* sera specifically precipitate gp36 from cell extracts. Although the anti-gp52 serum is highly specific for MMTV encoded components, it co-precipitates a small proportion of gp36 (see also Results). Affinity-purified fluorescein-labeled antibodies specific for rabbit or goat IgG were obtained from Litton Bionetics, Inc. (Kensington, MD).

Other Methods: Methods for labeling cell cultures with L-[^{35}S]methionine, for immunoprecipitating proteins from cell extracts or from culture media, and for electrophoresing proteins in polyacrylamide gradient gels in the presence of SDS and relative molecular weight standards have been described (19). For this labeling, cultures in 25-cm² flasks were thoroughly washed and then labeled in 5 ml of medium containing 100 μCi L-[^{35}S]methionine (1 Ci/mmol). Control studies established that the quantities of radioactivity incorporated by these cultures were directly proportional to the specific activities of the L-[^{35}S]methionine added onto the cell layers. Cell surface proteins were labeled with ^{125}I by lactoperoxidase-catalyzed iodination (12). Methods for coating sheep erythrocytes with *Staphylococcus aureus* protein A and for rosetting viable cultured cells in the presence of antisera reactive with cell surface antigens have been described (5, 19). Similar results were obtained using immunobeads (Bio-Rad Laboratories, Richmond, CA) coated with affinity-purified antibodies to immunoglobulins. Indirect fluorescent antibody studies were done using either viable cells on coverslips incubated in culture media or after fixation for 10 min at 0°C with methanol. The fluorescence studies of viable cells (Fig. 2) were performed using a Leitz Diavert microscope equipped with epifluorescence and phase-contrast optics generously provided by, and used with the guidance of Dr. William Woodward (Oregon Health Sciences University). Quantitative analyses of band intensities in autoradiograms was also described previously (18). Preparation of culture dishes coated with poly(2-hydroxyethylmethacrylate) (Aldrich Chemical Co., Milwaukee, WI) was also described earlier (8).

RESULTS

MMTV-infected M1.54 Cells Appear to Contain Different Amounts of Virus-encoded Cell Surface Glycoproteins

Fig. 2 shows a rosetting analysis of a freshly prepared subclone of M1.54 cells grown with dexamethasone. Binding of protein A-coated erythrocytes to the cell surfaces occurs in the presence of antisera specific for the MMTV glycoprotein gp36 (Fig. 2, *top*) or gp52 (*middle*) but not in the presence of normal serum (*bottom*). Similarly, rosetting is absent from

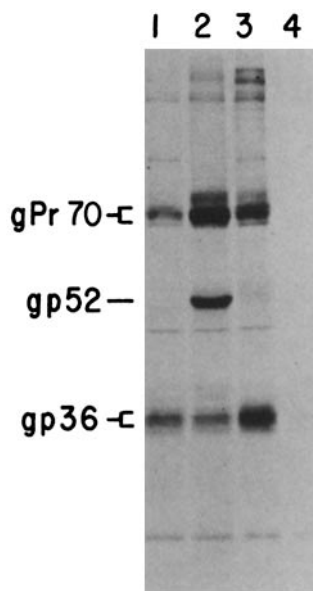


FIGURE 1 Characterization of antisera by immunoprecipitation of L-[^{35}S]methionine-labeled proteins from cell extracts, followed by electrophoresis of the proteins in polyacrylamide gels in the presence of 0.1% SDS. M1.54 cells were labeled for 2 h with L-[^{35}S]methionine before preparation of the cell extract. Aliquots of the extract were precipitated in the presence of Pansorbin with antisera, or with preimmune rabbit serum or normal nonimmune goat serum. Lane 1, the rabbit anti-*env* antiserum. This precipitates the gPr70 precursor and gp36. Lane 2, goat anti-gp52. This co-precipitates a variable but usually small proportion of the gp36 (see also Figs. 8–10).

Lane 3, goat anti-gp36. Lane 4, preimmune rabbit serum. Similarly, the MMTV encoded components were not precipitated by the normal goat serum (see Fig. 7).

uninfected cells or from infected cells incubated for 3 d without dexamethasone (see below). However, the specific rosetting is extremely heterogeneous—some cells label strongly whereas others are barren.

Docking of erythrocytes presumably requires cooperative formation of clustered bonds, and it conceivable that all cells contain the antigens but that clustering can occur only on certain cells. However, diversity was also observed when the cell cultures were labeled with fluorescent antisera. For example, Fig. 3 shows an analysis of gp52 antigens on the surfaces of viable M1.54 (subclone 2) cells. Only some of the cells are labeled. Moreover, the fluorescence is rather uniformly deposited over the surfaces of reactive cells at 4°C (Fig. 3, middle panels) whereas it becomes cross-linked into patches when the labeling is done at 25°C (upper panels). We conclude that gp52 is mobile when the plasma membrane lipids are in a fluid state.

Plasma Membrane Diversity Correlates with Differences in Cell Anchorage to the Substratum

Several observations indicate that the cell surface diversity is caused by a microenvironmental parameter rather than by heritable differences in MMTV gene expression. First, as mentioned above, the heterogeneity occurs in freshly isolated subclones. Second, the proportion of rosetted cells is dramatically affected by cell density (see Fig. 4). It is very low when the cells are isolated or in small colonies with ample space to flatten and migrate (Fig. 4, *top*), but it rises to 100% when the cells become crowded (*bottom*). Consistent with these observations, individual large colonies rosette heterogeneously in a target-like manner. The crowded central colony regions contain round, less anchored cells that rosette extensively, whereas the peripheral less crowded colony margins contain relatively adherent barren cells (see Fig. 5, *top*).

The above observations would be compatible with different interpretations because crowding causes intercellular contacts, reduction in anchorage, and inhibition of cell migrations.

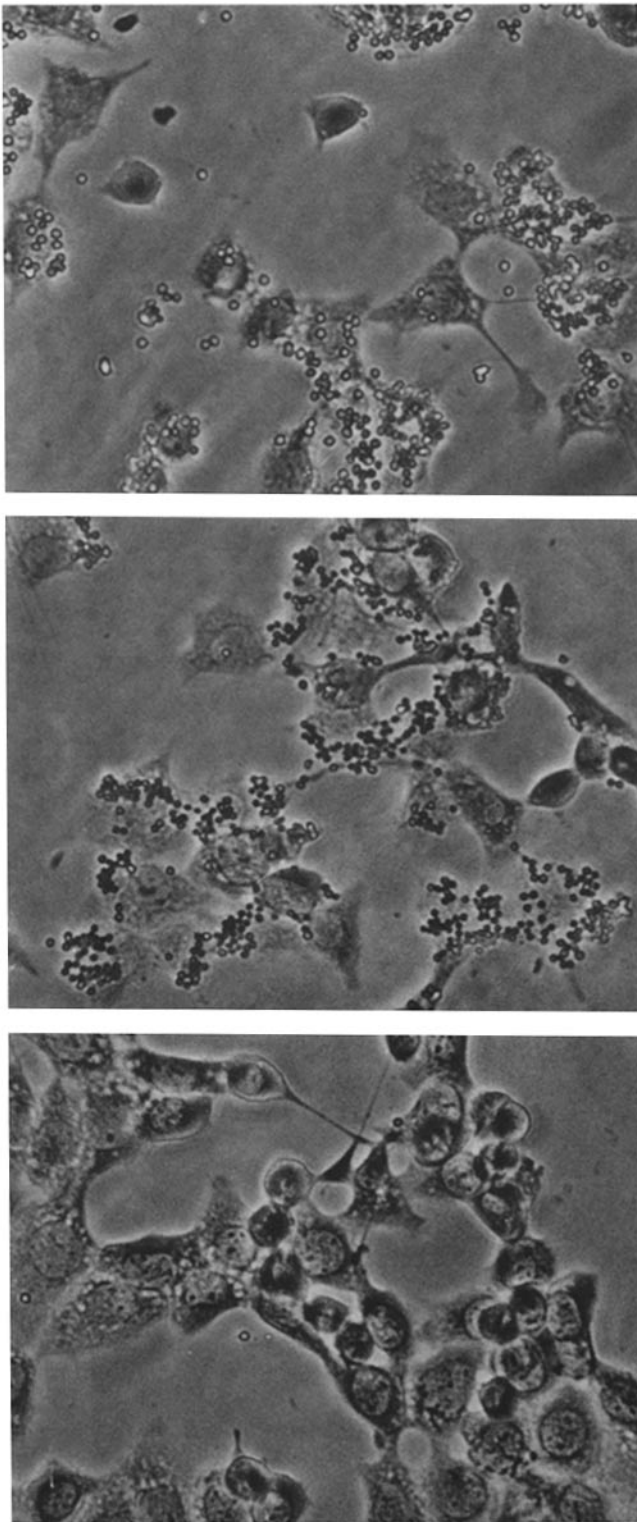


FIGURE 2 Heterogeneity of rosetting for MMTV-encoded cell surface antigens. A freshly isolated subclone of M1.54 cells (clone 2) was plated at 3×10^5 cells in a 25-cm² flask and was grown for 3 d in the presence of $1 \mu\text{M}$ dexamethasone. Cultures were subsequently reacted with protein A-coated sheep erythrocytes in the presence of antiserum specific for gp36 (top), gp52 (middle), or normal goat serum (bottom).

Moreover, crowded cells might divide less frequently because of either cell contacts or rounding with attendant reduction of anchorage (8). However, we have isolated a weakly adherent

variant of M1.54 cells (i.e., clone 6). Colonies of this variant grow in a relatively open lattice-like fashion in which all cells have a rounded morphology. Moreover, all clone 6 cells rosette extensively, even when growing in isolation or at the peripheral margins of colonies (see Fig. 5, bottom). Furthermore, the clone 6 colonies grow as rapidly as the M1.54 parental colonies. This implies that the critical microenvironmental parameter that causes surface expression of MMTV glycoproteins is unlikely to be intercellular contacts or reduced mitotic activity. Rather, it most closely correlates with a loss of anchorage indicated by a round morphology.

Additional evidence supporting this conclusion was obtained using culture dishes coated with poly(2-hydroxyethyl-methacrylate) to reduce adherence. This caused the M1.54 cells to round up and accumulate MMTV glycoproteins in their plasma membranes (see Fig. 6). Although the cells also tended to cluster together on these dishes, isolated cells were also all rosetted.

Total Cellular Content of MMTV Glycoproteins As Seen By Fluorescent Antibody Staining of Fixed M1.54 Cells

The previous results might occur if MMTV glycoproteins were restricted to the basal surfaces of highly adherent cells but could diffuse onto the apical surfaces of less anchored cells (15). However, lifting cells from culture dishes with EDTA was not rapidly followed by a change in the diversity of cell labeling with extracellular antibodies (unpublished observations).

Fluorescent antibody studies of fixed M1.54 cells also indicated that the cells contain different quantities and intracellular localizations of MMTV glycoprotein antigens. Fig. 7 shows a representative double labeling analysis of M1.54 cells that were rosetted on their surfaces before fixation with methanol and subsequent fluorescent antibody staining. The cells fluoresce to different extents, and appropriate controls suggest that the fluorescence is a specific indicator of MMTV glycoproteins. Also, there is a strong correlation between fluorescence and rosetting, since the rosetted cells tend to be highly fluorescent and vice versa.

Although the nonrosetted cells are relatively less fluorescent, they fluoresce much more than control uninfected cells or infected cells cultured in the absence of dexamethasone or reacted with preimmune serum. The specific fluorescence of the nonrosetted cells has an intracellular distribution, which suggests that antigen is localized primarily in the Golgi apparatus (perinuclear focal fluorescence that is generally oriented away from points of cell-cell contact [3]) and endoplasmic reticulum (punctate disposition throughout the cytoplasm). Confirmation of Golgi zone localization was obtained by a double-labeling analysis using rhodamine-labeled wheat germ agglutinin (this binds to complex oligosaccharides in the Golgi apparatus and on cell surfaces [23]) and fluorescein-labeled antisera to the MMTV glycoproteins (unpublished observation). In addition, the proportion of antigen in the Golgi zone of these nonrosetted cells appears higher for gp52 than for gp36 (unpublished observation), consistent with a model for gp36 degradation described below. These results support evidence given in the next section that MMTV glycoproteins are synthesized in all M1.54 cells but that their turnover rates, steady-state intracellular concentrations, and subcellular localizations are subject to modulation.

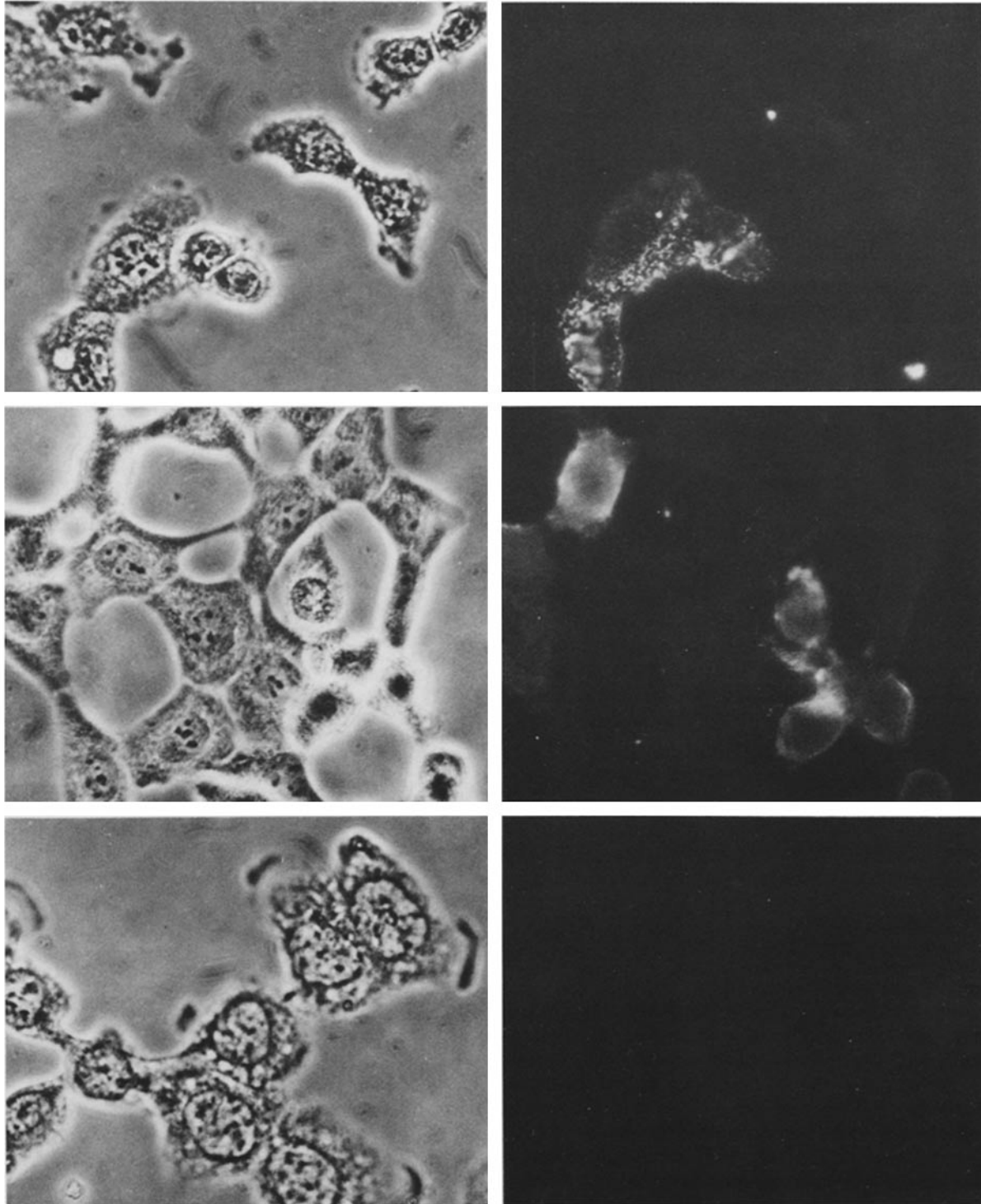


FIGURE 3 Heterogeneity of fluorescent antibody assay for MMTV-encoded cell surface antigens. After M1.54 (clone 2) cells were grown on coverslips in the presence of $1 \mu\text{M}$ dexamethasone, the viable cells were reacted for 1 h with anti-gp52 (top and middle) or normal goat serum (bottom). After they were washed with culture medium to remove unadsorbed serum, the cells were reacted for 1 h with fluorescein-labeled rabbit anti-goat immunoglobulins. After they were washed further, the labeled cells were fixed with methanol. Representative fields were photographed when viewed first by phase-contrast microscopy (left) and then by fluorescence microscopy (right). The upper panels show cells reacted with anti-gp52 and with fluorescent anti-immunoglobulins at 25°C . The antigen is patched. The middle panels show cells reacted at 0°C . The antigen is more uniformly distributed on the surfaces of reactive cells. The lower panels show cells reacted at 25°C but with normal goat serum instead of anti-gp52. All of the cells on this coverslip were unlabeled.

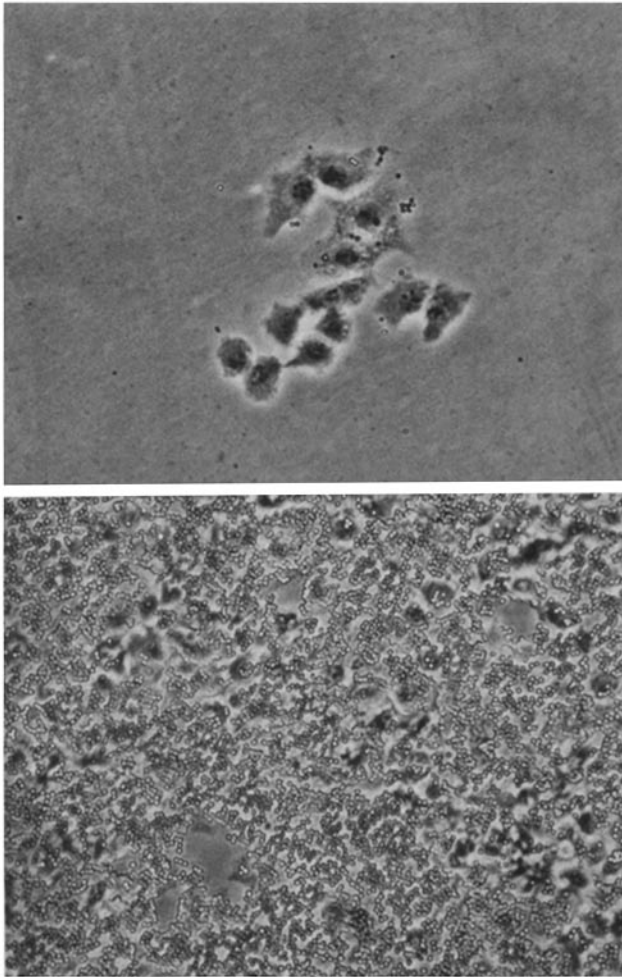


FIGURE 4 Rosetting assay for MMTV-encoded antigens on the surfaces of cells grown at either low or high cell densities. M1.54 cells were continually grown in $1 \mu\text{M}$ dexamethasone and were plated into 25-cm^2 flasks at either 10^4 or 10^6 cells/flask. After 4 d in the same culture conditions, the cells were reacted with protein A-coated erythrocytes in the presence of anti-*env* antiserum, which reacts with gp36 (see Materials and Methods). Control flasks reacted with normal rabbit serum were unlabeled (data not shown). The upper panel shows a typical small colony of cells growing in conditions of sparse plating. The cells are almost completely unlabeled. The lower panel shows the heavy labeling typical of cells growing in crowded conditions.

Differences Between MMTV Glycoprotein Processing in Highly Anchored Flat Versus Rounded M1.54 Cells

The primary approach we have used to study this issue has been comparing the metabolism of MMTV glycoproteins synthesized either by high anchored flat cells growing in sparse conditions or by cells induced into rounded morphologies by growth at high cell densities (e.g., as in Fig. 4). Cells under these conditions were pulse-labeled with $L\text{-}[^{35}\text{S}]\text{methionine}$ and then chased with an excess of nonradioactive methionine. At various times, cells were lysed and aliquots containing equal numbers of lysed cells were precipitated with antisera, and the immunoprecipitated proteins were then analyzed by electrophoresis (see Materials and Methods). Fig. 8 shows an analysis of the radioactive glycoproteins recovered from cell

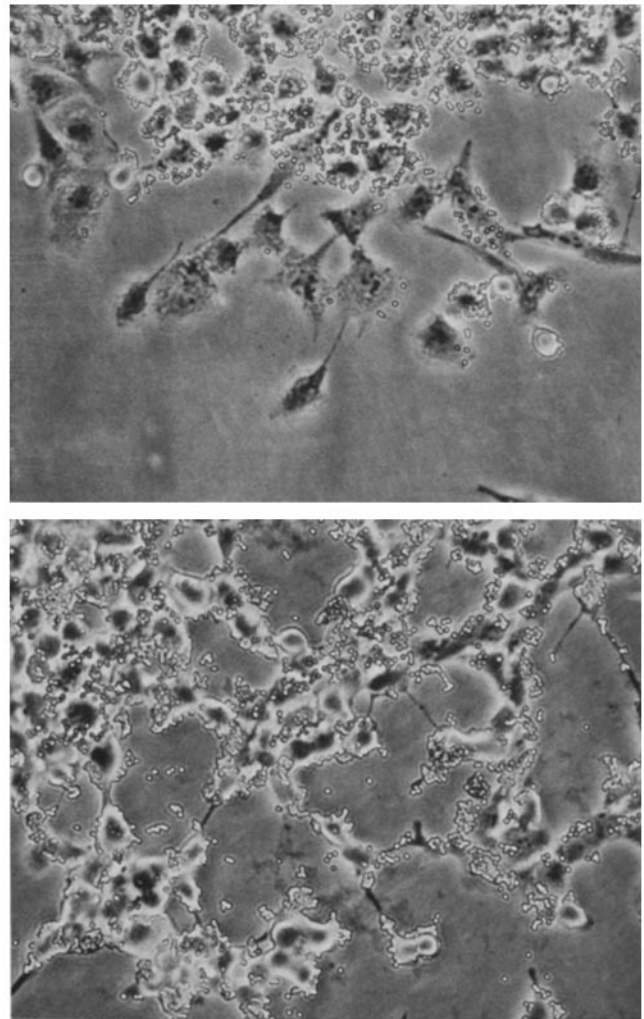


FIGURE 5 Rosetting of M1.54 cells growing as large colonies. 1×10^3 M1.54 cells were plated into 25-cm^2 culture flasks in the presence of $1 \mu\text{M}$ dexamethasone. After 15 d the colonies were reacted with protein A-coated sheep erythrocytes in the presence of antiserum specific for gp36. The upper panel shows a typical M1.54 cell colony after rosetting with the antiserum. The cells in the central colony region (upper portion) are heavily rosetted whereas the cells at the colony border are relatively flattened and mostly unlabeled. The lower panel shows the rosetted border of a mutant colony. The M1.54/clone 6 mutant cells attach only weakly to the substratum and the cells remain rounded even at the colony border. The center of the colony is toward the top. The clone 6 mutant cells rosette extensively even at the colony edges.

extracts in a representative experiment. Consistent with earlier reports, the MMTV glycoproteins are initially synthesized as an M_r 70,000 precursor glycoprotein (gPr70) that is subsequently processed into two smaller glycoproteins gp52 and gp36, which form a complex on the cellular and virion surface membranes (e.g., reference 20). In addition, some gPr70 is processed to form a secretory glycoprotein gp75 (20). However, there are several major differences between the two types of cell cultures. Most striking, as seen by their rates of disappearance during the cold chase, both gp36 and gp52 turn over much more slowly in round cells than in flat cells (compare the gp36 components in Fig. 8, left panel, lanes 1–5 with lanes 7–11; and the gp52 components in the right panel, lanes 1'–5' with lanes 7'–11'). A quantitative densitometric analysis

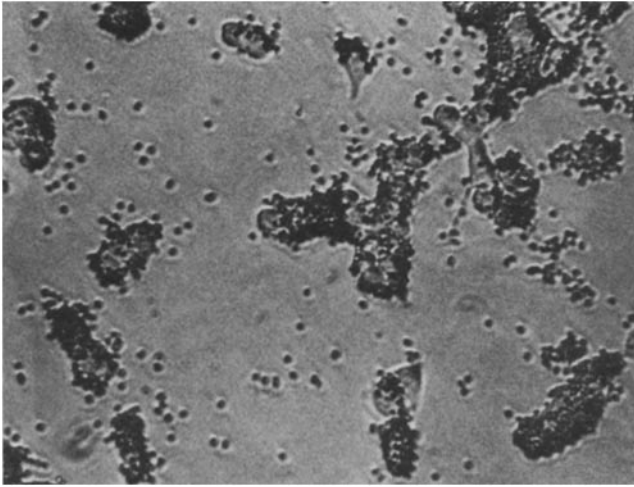


FIGURE 6 Rosetting of M1.54 (clone 14) cells grown on a poly(2-hydroxyethylmethacrylate)-coated surface. 1×10^4 cells were plated into 2-cm² culture wells in the presence of 1 μ M dexamethasone. After 24 h the cells were reacted with protein A-coated erythrocytes in the presence of antiserum specific for gp52. Because unbound erythrocytes were removed only by gentle rinsing to avoid detachment of weakly adherent rosetted cells, the erythrocyte background is relatively high in this experiment. The cells are only weakly anchored and they are uniformly rosetted. Control cultures in plastic dishes and with normal nonimmune serum gave results as in Fig. 1.

of the band intensities in this experiment implies that the half-lives for gp55 and gp36 turnover are \sim 25 min in flat cells, as compared with \sim 5 h in round cells.

Fig. 9 shows an analysis of the radioactive glycoproteins released into the culture media during this same experiment. Clearly, both gp36 (Fig. 9, lanes 1–3) and gp52 (lanes 1'–3') were released into the culture medium of round cells. However, although both these glycoproteins have short intracellular lives in flat cells (see Fig. 8), flat cells do not secrete any gp36 into their culture media (lanes 4–6), although these same media contain large amounts of gp52 (lanes 4'–6'). Consequently, gp36 must be rapidly degraded within flat cells. Quantitative densitometric analysis of these results and normalization of the data on the basis of equal cell numbers indicated that the round cells release relatively more gp75 but two- to threefold less gp52 per cell than do the flat cells.

Consistent evidence was obtained in independent repeats of the above experiment. Moreover, the labeling conditions were designed to eliminate methionine pool effects (see Materials and Methods). By quantitative evaluation of all results including studies in which the pulse-labeling time was only 15 min, we conclude that the cells in the two types of culture conditions synthesized similar quantities of the MMTV-encoded gPr70 precursor glycoprotein. Chemical analyses indicated that the cells growing in crowded and sparse conditions also contain similar quantities of DNA, RNA, and protein (unpublished results).

We have made several other pertinent observations. The gp36 released from poorly anchored round cells is apparently shed as a membranous virus-like particle that also contains some gp52. The latter particle was quantitatively pelleted from culture media by centrifugation at 159,000 g for 1 h, and it could be disrupted with 0.5% Triton X-100 detergent. However, no other shed MMTV glycoproteins, including the gp52 released from flat cells, were sedimented in these conditions.

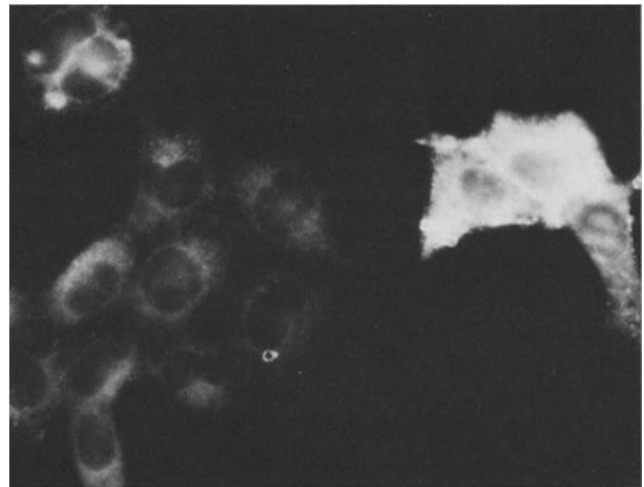
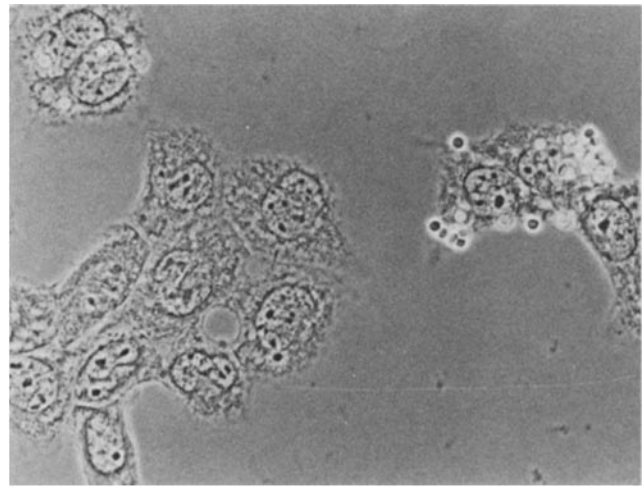


FIGURE 7 Double labeling assay for cell surface and for intracellular gp36 antigens. A culture of M1.54 cells was grown on coverslips for 3 d in the presence of 1 μ M dexamethasone before rosetting with protein A-coated erythrocytes plus the *env* antiserum that reacts with gp36. Only certain cells became rosetted. The washed cells were then fixed with methanol for 10 min at 0°C, and the fixed cells were reacted first with the *env* antiserum and then with fluorescein-labeled antiserum made to rabbit immunoglobulins. Top, phase-contrast microscopy of a representative field. Middle, fluorescence microscopy of the same field. Bottom, fluorescence microscopy of a typical field from a control coverslip that contained M1.54 cells cultured without dexamethasone. The cells appear unlabeled. Similarly, labeling was absent when we used uninfected HTC cells or infected cells reacted with preimmune serum (data not shown).

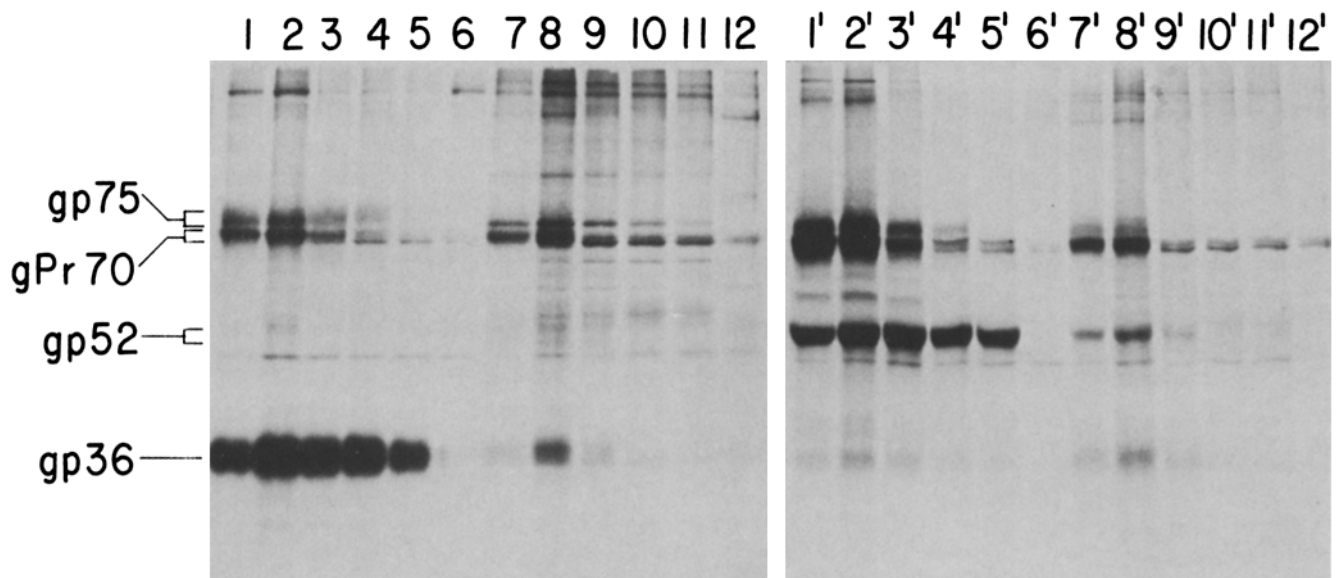


FIGURE 8 Synthesis and intracellular turnover of MMTV-encoded glycoproteins. M1.54 (clone 2) cells were plated into 25-mm² flasks in the presence of 1 μ M dexamethasone either at low density (5×10^4 cells/dish) or high cell density (2×10^6 cells/dish). After 3 d, the cultures were examined to confirm that the low density cells were sparsely plated and flattened whereas the high density cells were crowded and rounded. The cultures were washed thoroughly by incubations with 37°C labeling medium and were then labeled for 1 or 2 h with L-[³⁵S]methionine (see Materials and Methods). After 2 h of labeling, the remaining cultures were washed thoroughly by incubations with complete medium containing a large excess of unlabeled methionine, and the chased samples were harvested after additional incubations of 2, 5, or 12 h. Cell lysates from the low and high density cultures were adjusted to contain equal numbers of lysed cells, and aliquots were used for immunoprecipitations using either antibody to gp36 (left) or antibody to gp52 (right). The samples were then analyzed by electrophoresis followed by fluorography to detect the radioactive components. Lanes 1–6 contain the crowded cells plated at high density, and lanes 7–12 contain the flattened cells plated sparsely at a low density. Lanes 1 and 7, 1-h pulse; lanes 2 and 8, 2-h pulse; lanes 3 and 9, 2-h pulse followed by 2-h chase; lanes 4 and 10, 2-h pulse followed by 5-h chase; lanes 5 and 11, 2-h pulse followed by 12-h chase. Lanes 6 and 12 (left) are control precipitations from the 2-h lysates that were done with normal goat serum instead of antiserum. Lanes 6 and 12 (right) are similar control precipitations done using the 5-h chase lysates.

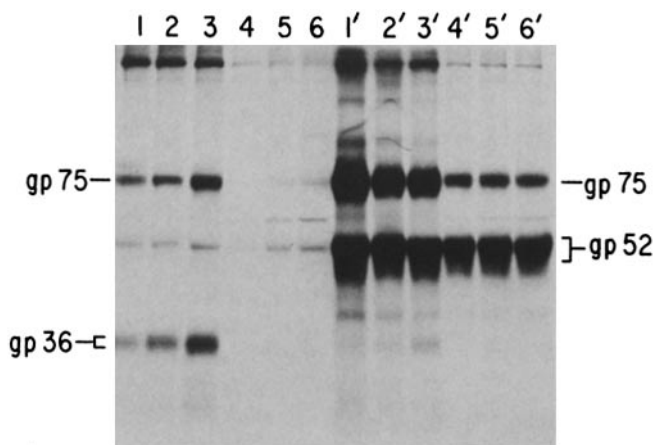


FIGURE 9 Shedding and secretion of MMTV encoded glycoproteins into the media of cultured M1.54 (clone 2) cells. The experiment is the same as in previous Fig. 8, except in this case the media recovered after the cold chase times (2, 5, and 12 h) were analyzed. The media from the high density cultures were diluted twofold before use. Equal volumes of all media were then immunoprecipitated using antibody to gp36 (lanes 1–6) or gp52 (lanes 1'–6'). Lanes 1–3, media from the high density cell cultures chased with unlabeled methionine for 2, 5, and 12 h and immunoprecipitated with anti-gp36; lanes 4–6, corresponding media from the low density cell cultures; lanes 1'–3', same media as lanes 1–3 but precipitated with anti-gp52; lanes 4'–6', same media as lanes 4–6 but precipitated with anti-gp52. No gp36 was detected in lanes 4–6, even when the fluorograph exposure time was increased 10-fold.

This is consistent with the hypothesis that round cells contain gp36/gp52 complexes in their plasma membranes that are shed by virion budding, whereas flat cells release gp52 only as a secretory component. Second, consistent with earlier studies (e.g., reference 20), we observe some gp75 synthesis and secretion by M1.54 cells (Figs. 8 and 9). Although gp75 contains both gp52 and some gp36 antigens, it precipitates relatively poorly with our antisera to gp36. Moreover, the ratio of gp75/gp52 in secretions is reproducibly higher from round than from flat cells (e.g., Fig. 9).

In addition, the gp36 synthesized in flat cells is reproducibly slightly larger (~1–2 kD) than that made in round cells. This was clearly seen when samples from flat and round cells were electrophoresed in alternating adjacent lanes. Recently, Firestone et al. (6) reported that M1.54 cells synthesize a small amount of gPr70 in the absence of dexamethasone and that this is not processed into plasma membranes because of an apparent rapid degradation of gp36. It is interesting that the larger gp36 is always formed under conditions that lead to its rapid degradation (i.e., in round cells lacking dexamethasone and in flat cells in the presence or absence of dexamethasone). Consequently, the rate of gp36 degradation may be controlled by a prior modification or signal that slightly alters its size.

However, the gp75 components were clearly evident in these lanes, which confirms that the immunoprecipitations were effective. gp75, however, apparently lacks some gp36 antigens (20), and it is inefficiently precipitated by our anti-gp36 serum.

Cell Surface Iodination

Fig. 10 shows an electrophoretic analysis of cell surface proteins that were labeled by iodination of viable cells with ^{125}I . Consistent with our immunocytochemical evidence (Fig. 4), two separate subclones of M1.54 cells that were tested both contain iodlatable gp36 and gp52 only when grown at high cell densities that caused rounding (left panel, lanes 2, 4, 6, 8) but not when grown with ample anchorage at low cell densities (lanes 1, 3, 5, 7). The right panel shows an electrophoretic analysis of the total ^{125}I -labeled plasma membrane proteins in these same cell extracts. The proportions of labeled cell surface components were reproducibly different in flat and round cells. For example, there seem to be unique components labeled in the two conditions of cell growth (e.g., component X) and also substantial differences in proportions of compo-

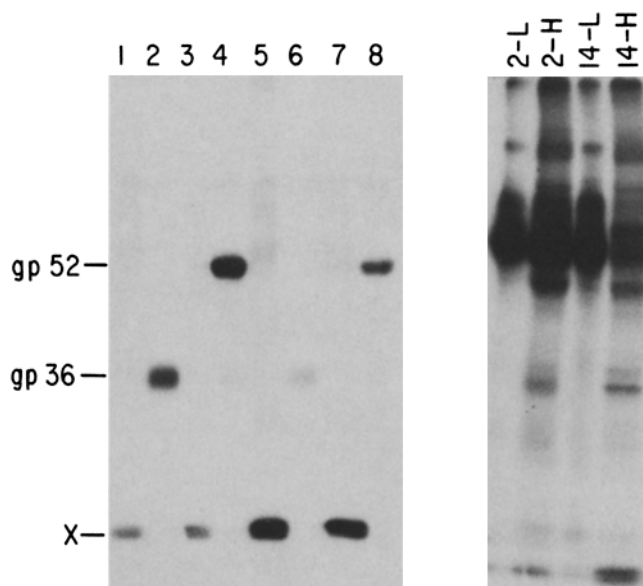


FIGURE 10 Iodination of proteins on the surfaces of cells grown at either low or high confluencies. M1.54 cell clones 2 and 14 were both grown in $1 \mu\text{M}$ dexamethasone in 25-cm^2 flasks at either low confluence (2×10^5 cells per flask) or until the cells were highly crowded (4×10^6 cells per flask). The cultures were then labeled with ^{125}I by lactoperoxidase-catalyzed iodination. The cell lysates from the low and high density cultures were adjusted to the same concentration of lysed cells. Lanes 1–8 show an electrophoretic analysis of proteins immunoprecipitated from the cell lysates of clone 2 cells (lanes 1–4) or clone 14 cells (lanes 5–8). Lanes 1 and 2, low versus high density lysates of clone 2 cells precipitated with anti-gp36; lanes 3 and 4, the same lysates precipitated with anti-gp52; lanes 5 and 6, low versus high density lysates of clone 14 cells precipitated with anti-gp36; lanes 7 and 8, the same lysates precipitated with anti-gp52. Component X co-precipitates nonspecifically with the Pansorbin even when normal goat serum is used (data not shown); however, it occurs on the surfaces only of the low density cells and is therefore present only in lanes 1, 3, 5, and 7. The right panel shows an electrophoretic analysis of the total ^{125}I -labeled cell extracts without immunoprecipitation. The lanes shown were in the same gel as those in the left panel, and the relative molecular weights therefore correspond. Lane 2-L, clone 2 cells labeled at low confluence; lane 2-H, clone 2 cells labeled at high confluence; lane 14-L, clone 14 cells labeled at low confluence; lane 14-H, clone 14 cells labeled at high confluence. The patterns of iodinated proteins are reproducibly and characteristically different for cells labeled at low versus high confluencies.

nents that appear to co-migrate. Clearly, the differences are not limited to MMTV-encoded components.

DISCUSSION

Plasma Membrane Expression of MMTV Glycoproteins Correlates with Loss of Cell Anchorage

Our immunocytochemical and metabolic results agree substantially, and they suggest that MMTV glycoprotein addressing in M1.54 hepatoma cells is regulated by a microenvironmental parameter that closely correlates with cell anchorage to the substratum. The glycoproteins are processed into the plasma membranes of round less anchored cells whereas they are rapidly degraded or secreted from cells that are flat and highly adherent to the culture dishes. When growing at moderate densities the cells apparently can migrate between crowded and sparser microenvironments and consequently alter their anchorage to the substratum. Correspondingly, their surface coats change, after a lag required for shedding or accumulation of glycoprotein components. Although the MMTV-encoded glycoprotein precursor is synthesized in similar quantities by all cells, the glycoprotein products accumulate in the cells to vastly different extents (e.g., see Figs. 7 and 8).

A Model for MMTV Glycoprotein Addressing in Epithelial Cells

Fig. 11 illustrates the major MMTV glycoprotein processing pathways in highly anchored flat versus round M1.54 cells. Our central proposal is that gp36 is a transmembrane glycoprotein with cytosolic and luminal domains and that it is degraded in the Golgi region or associated transition vesicles of flat cells, resulting in subsequent gp52 secretion as a soluble glycoprotein (see lower pathway in Fig. 11). On the contrary, such degradation is less extensive in round cells. In that case, the gPr70 precursor glycoprotein is predominantly cleaved between its gp36 and gp52 domains to yield gp36–gp52 complexes that accumulate on the cell surfaces and can be subsequently released in membranous vesicles by budding (see upper pathway in Fig. 11). Moreover, we propose that this same shift in efficiency of proteolysis also may cause enhanced gp75 secretion by the round cells. Consistent with previous evidence (20), we suggest that gp75 is derived from the gPr70 precursor by the addition of complex oligosaccharides and by proteolytic removal of the intramembranous and cytosolic gp36-related domains. Absence of these domains would explain gp75 solubility in aqueous media and its relatively weak reactivity with our gp36-specific antisera.

Recently, Firestone et al. (6, 7) showed that M1.54 cells in the absence of dexamethasone produce a basal low amount of MMTV glycoproteins that are not processed into plasma membranes. They concluded that there is a dexamethasone-dependent step in processing these glycoproteins. It is striking that their data for processing in the absence of dexamethasone appear identical to our observations that concern processing in highly anchored flat cells in the presence of dexamethasone. Accordingly, we conclude that the dexamethasone-dependent processing step (6) (upper pathway in Fig. 11) can function only if the cells are poorly anchored and round. On the contrary, MMTV glycoproteins are processed exclusively as

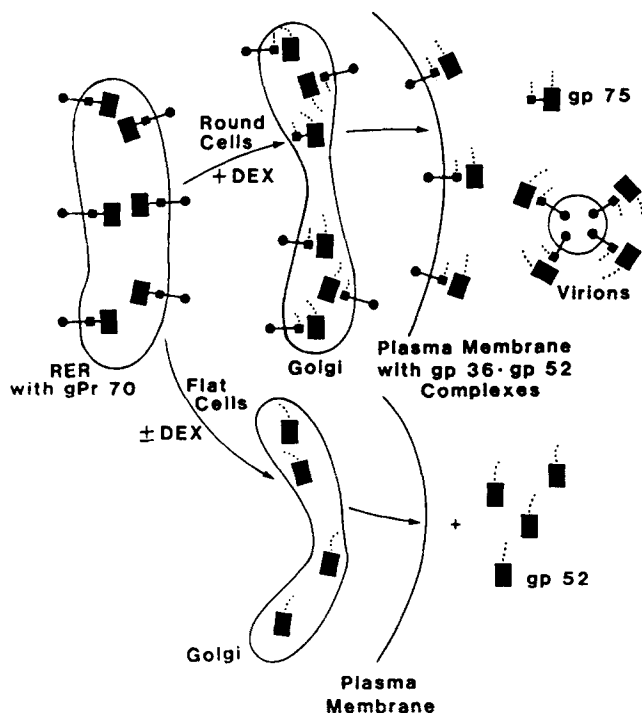


FIGURE 11 A model of MMTV glycoprotein processing in flat versus round cells. The gpPr70 precursor glycoprotein is co-translationally implanted into the rough endoplasmic reticulum (RER) with its gp52-related domain (large solid rectangle) and its gp36-related domains (small solid square and circle). As shown, the gp36 portion has both luminal and cytosolic subdomains since it spans the membrane. Complex oligosaccharide structures formed in the Golgi apparatus are depicted by chains of small dots. The critical effect of cell shape is to alter proteolytic cleavages of the gpPr70. In flat cells, as seen in the lower pathway, the gp36 domains are degraded, resulting in secretion of gp52. In round cells, as seen in the upper pathway, most of the precursor is cleaved in the Golgi zone or in transition vesicles to form gp36/gp52 complexes, which are subsequently transported to the plasma membranes. This form is later incorporated into virions that bud from the cell surfaces. However, some precursor in round cells is cleaved between the two gp36-related domains to produce secretory gp75. Based on the evidence of Firestone et al. (6), we propose that the upper pathway only occurs in round cells in the presence of dexamethasone. The lower pathway occurs in flat cells in all conditions and also in round cells in the absence of dexamethasone.

secretory components (lower pathway in Fig. 11) in flat cells under all conditions and in round cells deprived of dexamethasone. Based on these relationships, we propose that loss of cell anchorage causes a signal to be transduced to the intracellular organelles and that dexamethasone is required for this signal transduction system. The upper pathway in Fig. 11 can function only when the signal is received by the responsible organelles.

Do Similar Plasma Membrane Modulations Occur in Other Cells and Affect Other Glycoproteins?

Sarkar and Racevskis (20) observed heterogeneity of gp52 expression on the surfaces of three different mouse mammary tumor cell lines, and they also reviewed earlier evidence for this puzzling cellular diversity. Moreover, infected mammary epithelial *in vivo* often contain only trace amounts of MMTV glycoproteins, whereas explantation onto plastic culture dishes

causes diverse increases in cellular glycoprotein concentrations (11, 14). In infected mice, the MMTV glycoproteins are concentrated predominantly in progressive premalignant neoplasms (e.g., hyperplastic alveolar nodules) in which the epithelial histology is relatively disorganized (9, 11, 14, 21). These observations are all consistent with our results and they suggest that cell attachments may control MMTV glycoprotein processing in neoplastic mammary epithelia.

Our iodination data (Fig. 10) imply that the plasma membranes of flat versus round M1.54 cells may differ in their contents of cellular as well as MMTV-encoded proteins. Previous evidence also indicates that plasma membrane and secretory protein compositions can be controlled by cell anchorage (1, 10, 16, 26). These results and other evidence (see Introduction) are consistent with the hypothesis that the posttranslational control mechanism we have analyzed may be of general relevance. This could be important in embryogenesis and wound healing because it would enable cells to change their surfaces (and their corresponding responsiveness to factors that regulate mitosis or differentiation) in response to alterations in their adhesive connections. By this mechanism, for example, stable maintenance of small stem cell populations could be controlled simply by the size of an adhesive microenvironment rather than by a precise balance between mitogenic and differentiation-inducing factors. Some of the abnormal properties of cancer cell plasma membranes are apparently reversible consequences of reduced cell anchorage (16). Our results suggest that virus transmission and immunoresistance of tumor cells can also be influenced by cell anchorage. If these considerations prove correct, an ability to control the posttranslational processing switch could be useful. Our observation that in hepatoma cells this switch may depend upon glucocorticoids offers a promising approach for further investigations.

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