Glycoproteins of Coated Pits, Cell Junctions, and the Entire Cell Surface Revealed by Monoclonal Antibodies and Immunoelectron Microscopy

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ABSTRACT Topographical descriptions of three major plasma membrane glycoproteins of murine 3T3 cells were obtained by immunoelectron microscopy with monoclonal antibodies. A glycoprotein of $M_r$ 80,000 was distributed throughout the total cell surface. A second of $M_r$ 90,000 was concentrated in coated pits, and a third of $M_r$ 100,000 was localized at cell junctions.

The demonstrations of topographical domains on the cell surface have contributed to the understanding of cellular functions and the chemistry and biology of individual plasma membrane glycoproteins. The most extensively characterized of such domains are the clathrin-coated endocytotic vesicles (1) and junctional elements between cells (2, 3), both of which have unique protein compositions (4–9).

We previously described a number of cell surface proteins of murine cells (10), initially identified by use of monoclonal antibodies. Several of these were glycoproteins that have been purified and characterized biochemically (10–14). Each was a major cell surface component of the 3T3 mouse embryo cell line, with $10^4$ to $10^6$ antigenic binding sites per cell, and was present in a variety of differentiated cells of the mouse.

We have now used immunoelectron microscopy with monoclonal antibodies to determine the locations of these glycoproteins on the cell surface. The experiments were performed concurrently with the same cell line and antibodies of the same subclass. Distinct localizations of three glycoproteins to different sites on the cell surface were observed: coated pits, cell junctions, and the entire cell surface. These findings will provide a basis for additional studies of the chemistry and biology of the glycoproteins as major components of the cell plasma membrane.

MATERIALS AND METHODS

Antibodies: Rat anti-mouse fibroblast (AMF) monoclonal antibodies anti-glycoprotein 80 (anti-gp80 [AMF-15]), anti-gp90 (AMF-17), anti-gp100 (AMF-9), and anti-gp110 (AMF-14), as previously described (10), were used as hybridoma tissue culture supernatants. Each antibody was a IgG1 subclass.

Electron Microscopy: NIH/3T3 cells nonproductively transformed by Harvey murine leukemia virus (15) were grown at 37°C in 35-mm plastic dishes containing Dulbecco's modified Eagle's medium (Gibco Laboratories, Grand Island, NY) with glucose at 4.5 g/liter (DME), 10% (vol/vol) heat-inactivated fetal calf serum (Sterile Systems, Logan, UT), and 5% CO₂-95% O₂. In some experiments, monolayers at ~80% confluency were washed twice with DME for 5 min at room temperature. Cells were prefixed in 0.1% glutaraldehyde for 6 min at room temperature and washed twice with DME. Subsequent incubations were also at room temperature. Alternatively, monolayers were cooled to 4°C, washed twice with DME for 5 min, and maintained at 4°C throughout the antibody incubations. Cells, either prefixed or not, were incubated for 30 min with 2 ml of the monoclonal antibody in hybridoma culture supernatant, and then washed twice with DME. A second antibody, 10 μg/ml horseradish peroxidase conjugated goat anti-rat immunoglobulin (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD), was added in 1.5 ml of DME containing 1% fetal calf serum; after 30 min the cells were washed twice with DME. The cells were then fixed with 3% glutaraldehyde in 0.1 M sodium phosphate, pH 7.2, for 30 min on ice (16), and washed once with sucrose/phosphate buffer (0.2 M sucrose, 0.1 M sodium phosphate, pH 7.2). Excess aldehydes were neutralized by incubation for 10 min at room temperature with 300 mM glycine, 200 mM sucrose, 100 mM sodium phosphate, pH 8.0, and the cells were again washed with sucrose/phosphate buffer. The enzyme reaction mixture containing 0.5 mg/ml 3,3'-diaminobenzidine, 0.03% H₂O₂, 50 mM Tris-HCl, 2.7 mM KCl, 137 mM NaCl, 1.5 mM KH₂PO₄, 8 mM Na₂HPO₄, pH 7.6 (17), was added for 30 min at room temperature, after which the cells were washed once with sucrose/phosphate buffer. Cells were postfixed at 4°C for 30 to 45 min with 1.0% O₃O₂ in 100 mM phosphate buffer, pH 7.2. The specimens were dehydrated in 50%, 70%, and 95% cold absolute ethanol, consecutively, for 5 min each. They were then warmed at room temperature for 15 min, washed with fresh absolute ethanol at room temperature for 5 min, and rinsed for 5 min in Luft's mixture of Epon 812 (18). Fresh Epon mixture was added and the samples were polymerized at 60°C for 24 h. Pieces of the embedded cell cultures were cut with a jeweler's saw, mechanically separated from the culture dish fragment, and embedded in the Epon mixture in flat molds. Care was taken to cover the culture side of the embedment with additional resin. The specimens were polymerized as before and sectioned on...
a diamond knife. Some sections were poststained with uranyl acetate and lead citrate. Grids were viewed directly with a Zeiss EM 10.B operated at 60 kV with a 30 µm objective aperture.

RESULTS

Immunoelectron Microscopy

Immunoelectron microscopy with monoclonal antibodies was used to describe the topographies of three plasma membrane glycoproteins of mouse embryo 3T3 cells. The properties of the rat IgG2a, monoclonal antibodies selected for binding to major cell surface glycoproteins of NIH 3T3 mouse embryo cells have been described previously (10–14). As shown below, three different sites of glycoprotein localization were observed. A glycoprotein of M, 80,000 was abundantly distributed throughout the cell surface, another of M, 90,000 was localized primarily in coated pits, and one of M, 100,000 was concentrated at cell junctions.

Cell-binding sites of the monoclonal antibodies were visualized by means of horseradish peroxidase coupled to goat anti-rat immunoglobulin. Cell monolayers were maintained at 4°C during incubation with antibody to prevent redistribution and internalization of surface molecules. Alternatively, cells were fixed with 0.1% glutaraldehyde prior to antibody incubations to insure that the native distributions of the glycoproteins were maintained. Control studies were performed using tissue culture supernatant of the P3X63Ag8 mouse myeloma cell line, secreting a nonspecific mouse IgG, (19), and the AMF-14 antibody, a rat IgG2a monoclonal antibody against a M, 110,000 glycoprotein that was inaccessible to lactoperoxidase labeling on the surface of intact 3T3 cells (12) (Fig. 1). In the absence of specific antibody binding, the cell surface was completely free of electron-dense material, except for a low level of granular staining occasionally seen in poststained sections of endocytotic pits. The absence of label in regions of close association between cells and in endocytotic pits was particularly relevant in establishing the regional specificity of the glycoproteins that we examined.

Major Cell Glycoprotein of M, 80,000

The M, 80,000 glycoprotein recognized by monoclonal antibody AMF-15 was the predominant iodinated cell surface polypeptide of 3T3 cells (10). Immunoelectron microscopy showed that this glycoprotein was present throughout the surface of the cell (Fig. 2). All regions of the cell surface were intensely labeled, including microvilli, endocytotic pits, and regions of apparent cell-cell contact. Antibodies were present on both upper and lower aspects of the cell; however, the accumulation of label appeared to be greater on the distal surfaces of the cells as compared to underlying surfaces in multilayered layers. Possibly, access of the labeling reagents to the underlying areas was limited, or there was polarization in the concentration of this glycoprotein in an oriented cell culture. The results were the same with unfixed cells labeled at 4°C or with cells fixed with 0.1% glutaraldehyde prior to labeling at room temperature.

Coated Pit Glycoprotein of M, 90,000

The M, 90,000 glycoprotein recognized by monoclonal antibody AMF-17 was also a major constituent of the fibroblast plasma membrane (10). It was a highly acidic glycoprotein that was externally labeled with 125I. Immunoelectron microscopy showed that the M, 90,000 glycoprotein was localized primarily in coated pits (Fig. 3). Many cells contained several of the antibody labeled pits within a small region of the cell surface. Label was generally deposited precisely over the length of membrane occupied by clathrin on the exterior side of the cell membrane, but sparse label was also occasionally observed in regions of the membrane leading into coated pits. There was little evidence of staining elsewhere in the membrane. The distribution of the M, 90,000 glycoprotein was the same with unfixed cells labeled at 4°C or when cells were prefixed with 0.1% glutaraldehyde prior to labeling at room temperature. These facts suggest that redistribution of the M, 90,000 glycoprotein was effectively prevented and that the glycoprotein was intrinsic to the coated pits or prelocalized.

Cell Junction Glycoprotein of M, 100,000

A remarkably different labeling pattern was obtained with the AMF-9 monoclonal antibody that reacts with a glycoprotein of M, 100,000, another major constituent of the 3T3 plasma membrane components externally labeled with 125I (10). This glycoprotein was localized at regions where the membranes of adjacent cells were closely associated (Fig. 4).
FIGURE 2
Open expanses of the cell surface were largely devoid of label, while apposed surfaces exhibited multiple sites of antibody binding. Label was observed both along continuous stretches of associated membranes and at intermittent sites, giving the appearance of variably sized patches of cell-cell contact. Occasional isolated patches of label were found on membranes with no apparent cell contact sites; these were minor localizations and some sections may have been tangential to an apposed membrane. The accumulation of label often spanned distances of five- to tenfold greater thickness than the plasma membrane. Cells prefixed with 0.1% glutaraldehyde gave the same results as nonprefixed cells maintained at 4°C. The glycoprotein appeared to be absent from sites of cell-substratum association.

**DISCUSSION**

This study's most striking finding was the specific distribution of the Mr 90,000 coated pit and Mr 100,000 cell junction glycoproteins. This finding was unexpected, in that these glycoproteins are major cell surface constituents that could be expected to distribute throughout the cell plasma membrane as does the Mr 80,000 polymorphic glycoprotein and several other cell surface components (20–25). The observed localizations appear to represent most of the antigenic sites on the cell surface, although detection of other diffuse sites may not have been within the sensitivity limits of this assay. The possibility also exists that only divalent antibody binding at sites of high antigen concentration was detected, and that less stable monovalent interactions with isolated antigenic sites were not observed. We consider this situation very unlikely, since at saturating levels of antibody monovalent binding is likely to predominate, regardless of antigenic site density (26). In exceptional cases, if monovalent antibody-antigen complexes are unstable, divalent binding may predominate, given sufficient mobility of antigen in the membrane. In any case, these results reveal specific sites of antigen concentration that are likely to be relevant to the biological functions of the molecules.

The Mr 100,000 cell junction glycoprotein was previously identified by immunoprecipitation of cell surface proteins labeled biosynthetically with [35S]methionine or vectorially with 125I (10). The glycoprotein was markedly heterogeneous, with a number of components varying in isoelectric point between 7.0 and 5.7. The migration of the molecule in SDS-PAGE was the same in reducing and nonreducing conditions. The molecule was strikingly concentrated in regions of cell contact and was largely absent on the exposed surfaces of cells or on surfaces not closely associated with other cells. Light microscopic immunohistochemistry of mouse tissues showed a localization of the Mr 100,000 glycoprotein to surfaces of cells with prominent junctional complexes such as squamous and columnar epithelial cells and hepatocytes (Murphy, T. L., T. W. Bauer, and J. T. August, unpublished results). Ganglion cells of the autonomic nervous system were also intensely labeled and the antigen was detected in most of the brain. A suggested role of the glycoprotein is in cell-adhesion or cell communication. Certain well-defined junctional elements between cells have been described by morphologic or physical criteria (2, 3) and a number of proteins associated with such junctional structures have been described. Rat liver gap junctions contained a major component of Mr 28,000, with other polypeptides that were possibly aggregates or proteolytic fragments of the Mr 28,000 species (4, 6). Desmosome cores, consisting of the desmosomal intercellular material and associated plasma membrane regions purified from bovine muzzle, were enriched in proteins of Mr 150,000, 115,000, 100,000, and 22,000 (7). We have not yet characterized the ultrastructure of the site of glycoprotein 100 (gp100) localization in relation to these morphologically defined junctions because of the relatively light fixation conditions used to preserve antigenicity. Other cell surface molecules from both vertebrate and lower organisms have been shown to be involved in adhesion (27–35); a Mr 105,000 glycoprotein, cell-CAM 105, isolated from rat hepatocytes (29); N-CAM, found as a Mr 140,000–190,000 glycoprotein in adult mouse, rat, chicken, and human brain, or as a Mr 200,000–250,000 glycoprotein in embryonic tissues (30–32); a Mr 68,000 cell adhesion molecule of embryonic chicken hepatocytes (33); fibronectin, a Mr 200,000–250,000 molecule implicated in adhesion of embryonic and adult fibroblasts (34); and laminin, a Mr 220,000/440,000 complex in basement membranes (35). The properties of the presently described gp100 cell junction glycoprotein in relation to these other cell adhesion molecules remain to be demonstrated.

Another major constituent of the 3T3 cell surface labeled externally with 125I was an acidic glycoprotein of Mr 90,000 (10). Light microscopic immunohistochemistry showed a restricted expression of this antigen in certain differentiated cells of mouse tissues (Murphy, T. L., T. W. Bauer, and J. T. August, unpublished results). The Mr 90,000 antigen was most prominent on macrophages and lymphocytes. Staining for...
this glycoprotein was also observed in some intestinal epithelial cells, basal cells of some squamous epithelia and hepatocytes. The major surface localization of this glycoprotein was in coated pits. The results were the same with cells prefixed with glutaraldehyde, suggesting that the glycoprotein was intrinsic to coated pits or preclustered during growth of the cells. In this respect, gp90 is similar to the low density lipoprotein receptor, M, 164,000, and the asialoglycoprotein receptors which are primarily concentrated in coated pits, prior to binding of ligand (36, 37). Other receptors, such as those for α2-macroglobulin, epidermal growth factor, pseudomonas toxin, and some viruses, are diffusely distributed on the cell surface and join the coated pits after ligand binding (38, 39). The presence of gp90 in coated pits suggests that it may be a receptor. The gp90 was distinguished from the low density lipoprotein receptor, which has a similar pattern of cell surface localization, by molecular weight and the effect of neuraminidase treatment: The desialylated LDL receptor increased in mobility on SDS PAGE; in contrast, the mobility of the M, 90,000 glycoprotein decreased (40) (Freedly, J., and J. T. August, unpublished results). The M, 90,000 glycoprotein has a similar molecular weight to the transferrin receptor subunit, another prominent cell surface antigen; however, the transferrin receptor is a disulfide-linked molecule that shows an M, 180,000 under nonreducing conditions, whereas the M, 90,000 glycoprotein has never been observed as a disulfide linked dimer (41) (Hughes, E. N., J. Freedly, and J. T. August, unpublished results). An alternative role for the gp90 is that this glycoprotein may be a structural component of the coated pit. Purified coated vesicles from a variety of sources contain families of polypeptides of M, 100,000, 55,000, and 33,000–38,000, in addition to the major constituent, clathrin (8–9). A possibly related property of gp90 is an association with the cytoskeleton. Six independently derived monoclonal antibodies against the gp90 co-precipitated the major heat-shock-induced polypeptides of M, 70,000 and 72,000 (42). The tryptic peptides of these heat-shock proteins closely resembled those of a reported M, 68,000 cytoskeletal associated protein of HeLa cells (43, 44). A similar homology of heat-shock proteins and cytoskeletal components was reported by Wang et al. (45). The presence of gp90 in coated pits and the association of the glycoprotein with the cell cytoskeleton suggests that this cell surface component is involved in endocytosis, either as a receptor or as a structural component of an endocytotic vesicle.

The M, 80,000 glycoprotein was uniformly distributed on the surface of mouse embryo 3T3 cells. Bretsch et al. (46) using ferritin labeled AMF-12 antibody, also described the widespread distribution of this glycoprotein on 3T3 cells. In that study, gp80 appeared to be less concentrated in coated pits than in noncoated regions of the cell surface, perhaps because of steric hindrance due to the increased size of the ferritin label. Other molecules that are dispersed throughout the cell surface include theta-antigen, thymus-leukemia antigen, H-2 and HLA antigens, and surface immunoglobulins. These can be induced to redistribute into patches and caps after the addition of multivalent antibodies (20–25). The M, 80,000 glycoprotein was densely labeled with antibody, consistent with its high concentration on the cell surface. It was one of the most abundant known components of the 3T3 cell plasma membrane, accounting for 0.06% of the total detergent extracted protein of the cell, with over 106 antibody-binding sites per cell (12, 13). The glycoprotein was also present in high concentration in macrophages and subpopulations of bone marrow and blood polymorphonuclear cells. In leukocytes, the antigen was present on a molecule of about M, 92,000, as compared to the M, 80,000 antigen of 3T3 cells (13). The glycoprotein was genetically polymorphic, with the gene controlling its expression located on Chromosome 2, closely linked to the gene(s) for several other cell surface antigens: histocompatibility 3 antigen, lymphocyte m-11 antigen, lymphocyte 4 antigen, immune response 2 antigen, and β-2 microglobulin (12, 47). A large portion of gp80 appeared to be oriented extracellularly, since a water soluble fragment of M, 65,000 was cleaved from the cell surface by low concentrations of trypsin (13). This topology was similar to that of the HLA and H-2 molecules (48, 49).

The findings of this study significantly contribute to the understanding of the cell surface glycoproteins which were initially identified by monoclonal antibodies. Biological functions can now be considered for these proteins in combination with biochemical characteristics.

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