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This research is directed at a further understanding of the role of specific membrane glycoproteins in the cellular response to the environment, with particular reference to proteins acting in immune response mechanisms. We have focused on characterizing newly identified membrane glycoproteins whose biochemical properties provide evidence that they function in cell recognition, receptor, or response mechanisms. The major advance has been the identification and characterization of two glycoproteins that function specifically as principal components of the lysosomal membrane. The understanding of these membrane glycoproteins opens wholly new directions for studies of the mechanisms of lysosomal function in antigen presentation, processes involved in specific intraorganellar membrane flow, and the biogenesis of lysosomes. The studies have also encompassed the Ly-24 (Pgp-1) lymphocyte cell surface alloantigen, whose function appears to involve an association with plasma membrane proteoglycans and cell adhesion.

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# MOLECULAR BIOLOGY OF PROTEINS ACTING IN IMMUNE RESPONSE MECHANISMS

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# FINAL REPORT

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PRINCIPAL INVESTIGATOR: J. Thomas August

TITLE: Molecular Biology of Proteins Acting in Immune Response Mechanisms

## A. OBJECTIVE

The goal of this research is to expand our knowledge of certain newly characterized cell membrane glycoproteins that we have identified as having major roles in cell function, particularly in cells of the immune system. These investigations focus on the basic biochemistry of cell membrane proteins as it relates to the structure and function of these proteins. Our long-range objectives are to contribute to the understanding of specific biological phenomena at the molecular and biochemical levels and to improve our capacity to prevent or treat the consequences of exposure to infectious or toxic agents.

## B. RESEARCH PROGRESS

We have identified and characterized several previously unknown glycoproteins that are principal components of cell membranes and appear to play important roles in cell function. The primary advances during the grant period have been in the discovery and analysis of the major membrane glycoproteins of cell lysosomes and the application of these findings to studies of lysosomal biogenesis and function. Another significant advance has been the discovery of an association of the Pgp-1 (Ly-24) cell-surface differentiation alloantigen with cell membrane proteoglycans and cell adhesion processes.



### 1. LYSOSOMAL MEMBRANE GLYCOPROTEINS

#### BACKGROUND

The lysosome is an essential organelle which serves as the intracellular digestive system for virtually all eukaryotic cells and plays a critical role in the digestion and reconstitution of molecules and in antigen presentation. The constituents of its limiting membrane are assumed to be directly involved in many of the specialized functions carried out by this organelle, including (1) acidification of the intralysosomal compartment, (2) controlled release of the products of lysosomal digestion, (3) selective transport of particular molecules, and (4) specific interactions with other membrane organelles.

A great deal is known about the properties, biosynthesis, and targeting of the hydrolases found within the lysosomal lumen. However, the components of the lysosomal membrane have received much less attention and there are several major gaps in our understanding of the lysosome and related organelles: (1) The chemical and structural characteristics that account for the properties of the lysosomal membrane are unknown, and the proteins involved in specific lysosomal functions have not been identified. (2) It

is not understood how these specialized compartments are formed and maintained amidst the rapid and extensive exchange of membrane that occurs throughout the vacuolar membrane system. (3) The various types of specific cellular vacuoles have not been adequately defined.

In the course of this research, we have identified the major antigens of the lysosomal membrane by use of monoclonal antibodies; these antigens now include four proteins, two found in human and two in mouse cell lines. The study of the biochemical and molecular properties of these proteins should provide new insights into mechanisms involved in the function of this critical organelle.

PROGRESS

a. Two major lysosomal membrane glycoproteins of mouse cells, LAMP-1 and LAMP-2, have been identified, purified and characterized. This research represents the initial discovery of membrane glycoproteins specifically localized in lysosomes (Chen et al., 1985a, Chen et al., 1985b, Chen et al. 1986). Two membrane glycoproteins of cell lysosomes were identified, purified and characterized, LAMP-1 of $M_r$ 105,000 to 115,000 and LAMP-2 of $M_r$ 100,000 to 110,000. Both were major components of the lysosome: LAMP-1 was present in high concentration, representing about 0.1% of the total detergent-extracted cell protein; LAMP-2 comprised about 0.03%. Both molecules behaved as integral membrane components and were solubilized by detergent solutions and purified by antibody affinity chromatography.

b. The lysosomal localization of LAMP proteins was demonstrated by immunohistochemistry. The localization of these molecules in lysosomal membranes was demonstrated by immunofluorescent staining with monoclonal antibodies and by immunoelectron microscopy. Antibody binding occurred primarily at the limiting lysosomal membrane and was distinctly separated from colloidal gold-labeled alpha-2-macroglobulin accumulated in the lumen of the lysosome during prolonged incubation. LAMP-1 and LAMP-2 were also present in low concentrations on Golgi trans elements and certain multivesicular vacuoles but were not detected in receptosomes marked by the presence of newly synthesized alpha-2-macroglobulin or in other cellular structures.

c. The cell localization of LAMP-1 was distinguished from that of endocytosed alpha-2-macroglobulin and transferrin. Electron microscopic immunochemistry was used to compare the distribution of LAMP-1 to the distribution of alpha-2-macroglobulin and transferrin, at various times after their endocytosis (Goldenthal et al., 1988). At early times after the initiation of endocytosis, structures containing alpha-2-macroglobulin were not labeled by anti-LAMP-1, whereas at later times (>15 min) the same structures were strongly reactive with the antibody. In contrast, no LAMP-1 was detected at any time in vesicles containing transferrin. These data indicate that ligands which are internalized via coated pits and endosomes but are not destined for lysosomes (e.g., transferrin) do not traverse a lysosomal organelle compartment marked by LAMP-1. Ligands which are destined for lysosomal delivery, such as alpha-2-macroglobulin, reach a LAMP-1-positive organelle only after they traverse LAMP-1-negative vesicles.

d. The LAMP molecules were shown to be highly glycosylated: Studies of the biosynthesis and processing of LAMP-1 and LAMP-2 by pulse-labeling with [$^{35}$S]methionine showed that the proteins were synthesized as polypeptides of about $M_r$ 45,000, cotranslationally modified by the addition of about 20 high-mannose oligosaccharides to form precursor glycoproteins of about $M_r$ 90,000, and post-translationally processed by the addition of a heterogeneous mixture of complex-type oligosaccharides to form mature sialylated molecules of about $M_r$ 110,000.

e. The LAMP proteins were found to be rapidly delivered from the Golgi to lysosomes. The interorganellar movement of newly synthesized LAMP-1 in 3T3 cells was traced by pulse-labeling with [$^{35}$S]methionine, fractionation of subcellular organelles by density gradient centrifugation, and analysis of oligosaccharide processing (D'Souza et al., 1986). Synthesis and glycosylation of the core polypeptide in the rough endoplasmic reticulum were followed by processing from asparagine-linked high-mannose to predominantly complex-type oligosaccharides in the Golgi cisternae. The protein was then immediately delivered to subcellular fractions enriched in lysosomal enzyme activities. None of the newly synthesized protein was detected in the plasma membrane prior to delivery to lysosomes. Oligosaccharide processing and delivery to lysosomes was complete by 1 h after synthesis and the mature glycoprotein remained in a fraction containing lysosomal markers.

f. The $NH_2$-terminal amino acid sequences of the LAMP proteins have been determined. These sequences were used to synthesize oligodeoxy-nucleotide probes for the identification of corresponding cDNA clones.

g. cDNAs encoding LAMP-1 and LAMP-2 have been cloned and sequenced. We have used polyclonal antisera raised against the purified proteins and the oligodeoxynucleotides corresponding to the $NH_2$-terminal amino acid sequence to isolate the cDNA of LAMP-1 (Chen et al., 1988). The predicted structure of this protein (382 amino acids) included an $NH_2$-terminal intraluminal domain consisting of two homology units of approximately 160 residues each, separated by a proline-rich hinge region. Each homology unit contained four cysteine residues, with two intercysteine intervals of 36 to 38 residues and one of 68 or 76 residues. The molecule also contained 20 Asn-linked glycosylation sites within residues 1 to 287, a membrane-spanning region from residues 347 to 370, and a carboxyl-terminal cytoplasmic domain of 12 residues. A remarkable finding is that the biochemical properties and amino acid sequence of mLAMP-1 were highly similar to those of two other molecules that have been studied as cell surface onco-differentiation antigens: a highly sialylated, polylactosaminoglycan-containing glycoprotein isolated from human chronic myelogenous leukemia cells (Viitala et al., in press) and the mouse gp130 (P2B) glycoprotein (Laferte and Dennis, in press), in which an increase in β1-6 branching of Asn-linked oligosaccharides has been correlated with metastatic potential in certain tumor cells. The cloning and sequencing of cDNA of LAMP-2 is in progress.

h. Two lysosomal membrane glycoproteins of human myeloid cells, hLAMP-1 and hLAMP-2, have been identified, purified, and characterized. We have recently extended these studies to human cells in order to analyze the expression and function of these glycoproteins in cells of the immune system (Mane et al., submitted). Two human cell lysosomal membrane glycoproteins of ~120 kDa, hLAMP-1 and hLAMP-2, were identified by use of

monoclonal antibodies prepared against U937 myelomonocytic leukemia cells or blood mononuclear cells. The two glycoproteins were purified by antibody affinity chromatography and each was found to be a major constituent of human spleen cells, representing ~0.05% of the total detergent-extractable protein. Both molecules were highly glycosylated, being synthesized as polypeptides of 40 to 45 kDa and cotranslationally modified by the addition of Asn-linked oligosaccharides. $NH_2$-terminal sequence analysis indicated that each was ~50% identical to the corresponding mLAMP-1 or mLAMP-2 of mouse cells. Electron microscopic studies of human blood monocytes, HL-60, and U937 cells demonstrated that the principal location of these glycoproteins was intracellular, in vacuoles and lysosomal structures but not in the peroxidase-positive granules of monocytes. Transport of the proteins between organelles was evidenced by their marked accumulation in the membranes of phagolysosomes. A fraction of each glycoprotein was also detected on the plasma membrane of U937 and HL-60 cells but not on a variety of other tissue culture cells. This cell-surface expression may be differentiation-related, since the proteins were not detected in the plasma membrane of normal blood monocytes and their expression on U937 and HL-60 cells was reduced when the cells were treated with differentiating agents. Cell-surface expression of both glycoproteins was markedly increased in blood monocytes but not in U937 cells after exposure to the lysosomotropic reagent methylamine HCl, indicating differences in LAMP-associated membrane flow in these cell types.

i. <u>The human LAMP proteins were shown to be transported at normal rates and expressed at high levels in the inclusion bodies of mucolipidosis II (I-cell disease) cells</u>. The localization, expression, and transport of hLAMP-1 and hLAMP-2 were studied in mucolipidosis II fibroblasts (I-cells), which lack the enzyme N-acetylglucosamine phosphotransferase and are defective in the mannose 6-phosphate receptor-mediated transport of acid hydrolases to lysosomes (Sandoval <u>et al</u>., submitted). Immunofluorescence microscopy studies showed the accumulation of the hLAMP glycoproteins in cytoplasmic vesicles (inclusion bodies) covering most of the cytoplasm of the cells, and immunoelectron microscopy revealed that these vesicles commonly contained membrane structures or electron-dense homogenous material characteristic of secondary lysosomes. In contrast to the markedly reduced levels of lysosomal hydrolases seen in mucolipidosis II fibroblasts, these cells expressed 3-to 4-fold higher levels of the hLAMP glycoproteins than did normal fibroblasts. Analysis of the biosynthesis of the hLAMP glycoproteins in I-cells pulse-labeled with [$^{35}$S]methionine indicated that the molecules are processed in the Golgi system, are transported at normal rates to vesicles with the high density characteristic of lysosomes, and have chemical properties similar to those synthesized in normal cells. These experiments provide direct evidence that the transport and targeting to lysosomes of vesicles containing integral membrane glycoproteins occurs by a mechanism independent of the mannose 6-phosphate system for transporting acid hydrolases.

## 2. CELL SURFACE DIFFERENTIATION ALLOANTIGEN Ly-24 (Pgp-1)

### BACKGROUND

Our laboratory (Hughes et al., 1981) and that of Trowbridge et al. (1982) independently identified a major cell-surface glycoprotein of about 80 kDa as a differentiation alloantigen of mouse lymphocytes. This molecule, Pgp-1 (Ly-24), has recently been shown to be an important differentiation marker on peripheral T cells that is expressed in response to primary antigenic stimulation (Budd et al., 1987). Its expression therefore provides an effective means of identifying memory T cells.

Pgp-1 was found in high concentrations on 3T3 cells, macrophages and subpopulations of bone marrow and blood polymorphonuclear cells. It was the predominant lactoperoxidase-iodinated component of 3T3 cells, and saturation binding of $^{125}$I-labeled antibody indicated that there were about $10^6$ antigenic sites per cell (Hughes et al., 1981; Mengod et al., 1983; Hughes et al., 1983). Electron microscopy showed that it was present throughout the surface of the 3T3 cell, including regions of cell-cell and cell-substratum contact (Murphy et al., 1983).

Monoclonal antibodies against Pgp-1 reacted with the protein from NIH Swiss, C57BL and other mice, but not with that from Balb/c, DBA or CBA mice, identifying this molecule as an alloantigen (Hughes et al., 1981; Colombatti et al., 1982). A comparable finding was reported by Lesley and Trowbridge (1982) and Trowbridge et al. (1982). The alloantigens of lymphocytes were recently classified as members of an Ly series and the 80-kDa glycoprotein was designated Ly-24.

The gene controlling expression of the protein in the mouse, designated Pgp-1, was mapped to chromosome 2 by measuring antibody binding to cells of recombinant inbred strains of mice and backcross segregants (Colombatti et al., 1982). Closely associated with Pgp-1 are the genes for the H-3 transplantation antigens, Ly-4 differentiation alloantigen, Ir-2 immune response function, beta-2-microglobulin, and the erythrocyte alloantigen Ea-6.

Pgp-1 was identified as an intrinsic membrane component and the detergent-soluble membrane fraction containing the protein was purified 2000-fold to near homogeneity by antibody affinity chromatography (Hughes et al., 1983). The purified protein represented 0.06% of the total protein of the crude cell extract, or approximately $2 \times 10^6$ molecules per cell.

Trypsin digestion of the Pgp-1 after cell-surface labeling with $^{125}$I yielded an antigenically active, soluble glycopolypeptide of about 65 kDa that contained all of the $^{125}$I and carbohydrate, indicating the presence of a large extracellular domain in the intact protein.

Measurements of fluorescence recovery after photobleaching were performed on the antigen-antibody complex within the plasma membrane of the cell after labeling with monoclonal antibody conjugated to fluorescein (Jacobson et al., 1984a; Jacobson et al., 1984b). The lateral diffusion of the molecule was about 2 orders of magnitude slower than that of a lipid probe. It is possible that peripheral interactions of the glycoprotein, either cytoplasmic or extracellular, limit its mobility in the plasma membrane.

Immunofluorescence analysis of live cells in the presence of bound antibody showed a redistribution of the protein-antibody complex in motile cells, with a decrease in fluorescent staining toward the leading edge. This gradient increased markedly with time and did not occur in $G_0$ or confluent cells. The antibody was "patched" only by application of second antibody directed against the monoclonal antibody. These patches were often aligned with underlying actin stress fibers.

PROGRESS

a. A fraction of Pgp-1 was found to be substituted with glycosaminoglycans. In the continuation of our studies of Pgp-1, we have obtained strong evidence that the molecule forms the protein core of a plasma membrane proteoglycan. Monoclonal antibodies to Pgp-1 immunoprecipitated a discrete fraction of a partially purified 3T3 cell proteoglycan of 200-400 kDa containing both heparan and chondroitin sulfate. This reaction was specific, since other monoclonal antibodies of the same isotype precipitated no proteoglycan, and an antibody with specificity for a different membrane glycoprotein immunoprecipitated a completely different fraction of the cell proteoglycan. In addition, we have observed that monoclonal antibody capping of Pgp-1 results in the co-capping of chondroitin sulfate, suggested that Pgp-1 is the core protein of the proteoglycan. Most recently, we have purified the proteoglycan associated with Pgp-1 by DEAE cellulose chromatography and are further characterizing this molecule.

b. Anti-Pgp-1 inhibition of cell adhesion has been demonstrated. Preliminary findings suggest that Pgp-1 functions as a cell adhesion molecule. Fibroblasts dissociated by EDTA into single-cell suspensions rapidly form large aggregates in the presence of 1 mM $CaCl_2$. Formation of these aggregates was inhibited by monoclonal antibodies against Pgp-1 but not by a large number of antibodies against other cell-surface molecules. Our proposed studies include the further characterization of the possible role of this protein as a cell adhesion molecule acting through an association with cell-surface proteoglycan.

c. A similar glycoprotein of human cells with a role in cell adhesion has been identified. We have extended these studies to human leukocytes in order to test the function of the protein in assays of blood cell function. A molecule with properties similar to those of Pgp-1 has been identified. The remarkable finding is that monoclonal antibodies to the human glycoprotein cause human peripheral blood lymphocytes to aggregate. We propose to continue the studies of this molecule and its role in immune cell function.

## C. SUMMARY OF THE IMPORTANCE OF THE FINDINGS

During the past five years we have identified and characterized three types of cell-surface/cell membrane glycoproteins whose biochemical properties suggest that they play important roles in cell function.

The lysosome-associated membrane glycoproteins provide wholly new tools for the study of lysosomal biogenesis and the interorganellar movement of membrane glycoproteins, and for analysis of the functions of lysosomal membrane proteins.

The Ly-24 (Pgp-1) cell-surface alloantigen is now recognized as an extremely useful differentiation marker for peripheral T cells that have been activated in vivo and for activated subsets of CD4- and CD8-positive cells (Budd et al., 1987).

The antibodies and hybridomas used in our studies are available to all investigators and have been deposited in a monoclonal antibody bank sponsored by the National Institute of Child Health and Human Development for the purpose of facilitating research in cell development and differentiation.

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