THE HEPATIC ASIALOGLYCOPROTEIN RECEPTOR

Author: Alan L. Schwartz
Division of Pediatric
Hematology-Oncology
Dana-Farber Cancer Institute
Children's Hospital Medical Center and
Department of Pediatrics
Harvard Medical School
Boston, Massachusetts

Referee: Gilbert Ashwell
Laboratory of Biochemistry and Metabolism
Section on Enzymes and Cellular Biochemistry
National Institutes of Health
Bethesda, Maryland

I. INTRODUCTION

The hepatic receptor for asialoglycoproteins was initially demonstrated in the early 1970s by the seminal studies of Ashwell, Morell, and their colleagues during investigations of the physiology of ceruloplasmin metabolism (reviewed by Ashwell and Morell[1]). It was shown that removal of terminal sialic acid residues from native mammalian serum glycoproteins, thus exposing the penultimate galactose residue, initiated prompt removal of the galactose-terminal glycoprotein molecule from the circulation. The specificity of this process for the terminal galactose residue was defined by (i.e., cleavage or oxidation) alteration of this terminal residue with beta-galactosidase or galactose oxidase[2] as well as by enzymatic resialylation.[3] The rapid clearance of the galactose-terminal glycoprotein from the circulation resulted from hepatic sequestration. The ligand and degradation products were ultimately found witin the lysosomal fraction of hepatocytes.[4] Further investigations revealed that more than 20 plasma proteins are cleared from the mammalian circulation via the hepatic asialoglycoprotein receptor (reviewed by Ashwell and Harford[5]). Of these, the asialo derivative of orosomucoid (ASOR), alpha$_1$ acid glycoprotein, is the best studied.

The recognition of asialoglycoproteins within the serum and their removal into the liver defines the presence of a process dependent upon ligand specificity and with resultant physiological consequences, i.e., uptake of ligand into the hepatocyte. The property of specificity in ligand recognition and its consequent binding to the liver cell defines the existence of a specific binding site. However, the physiological sequelae subsequent to ligand binding (i.e., receptor-mediated endocytosis, see below) thus defines in pharmacologic terms a receptor.

The hepatic receptor for galactose-terminal glycoproteins has been variously termed the hepatic asialoglycoprotein receptor, hepatic galactose receptor, or galactose/*N*-acetyl galactosamine receptor by virtue of its ligand specificity. In addition, the term mammalian hepatic lectin has been used since the ability of the receptor to agglutinate erythrocytes depends upon terminal carbohydrate exposure of integral erythrocyte proteins.[6] This review will use the term asialoglycoprotein receptor (ASGP-R).

In addition to the ASGP-R identified in mammalian liver, other carbohydrate-specific receptors have been described. These include those specific for mannose/*N*-acetyl glucosamine, fucose, and 6-phosphomannose. These carbohydrate-specific receptors have recently been the subject of a number of reviews.[5,7,8] Based on earlier studies of

the clearance of various plasma glycoproteins and their derivatives,[9] Lunney and Ashwell[10] identified a receptor in avian liver which is analogous to the mammalian ASGP-R. This avian hepatic receptor is specific for glycoproteins terminating in *N*-acetyl glucosamine, which is the penultimate carbohydrate residue in the naturally occurring avian plasma proteins. Details of the physical and kinetic properties of this receptor have been reviewed recently by Ashwell and Harford.[5]

The present review will focus on the physical characteristics and properties of the hepatic ASGP-R and its role in receptor-mediated endocytosis of asialoglycoproteins. The normal physiology of this receptor system will be discussed herein. The reader should refer to a recent review by Ashwell and Steer[11] for a discussion of the role of the hepatic ASGP-R in various pathophysiologic states.

II. PHYSICAL CHARACTERISTICS AND PROPERTIES OF THE ASGP-R

Many of the physical and chemical properties of the ASGP-R have been studied in considerable detail. Three species, rabbit, rat, and man, have been utilized as the source of ASGP-R for most of these studies. In general, similar properties and characteristics exist among the ASGP-R from these species.

The ASGP-R is an integral membrane protein. The receptor was initially solubilized from liver plasma membranes with Triton® -X-100 and shown to retain binding activity.[12] Subsequently, Hudgin et al.[13] isolated the receptor from a Triton® -X-100 extract of a washed acetone powder of liver via affinity chromatography with ASOR. This resulted in a purification of 300-fold with 41% yield as judged by ^{125}I-ASOR binding activity. The isolated receptor preparation demonstrated a high degree of aggregation in the absence of detergent, resulting in oligomers of molecular weight >500,000 daltons as determined by gel filtration chromatography. However, in the presence of Triton® -X-100, the preparation demonstrated a single species of approximately 250,000 daltons (Table 1). In the presence of sodium dodecyl sulfate and beta mercaptoethanol, the rabbit receptor as analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis is composed of two subunits, 48,000 (A) and 40,000 (B) daltons with relative abundance of 1:2.[13,14] Andersen et al.[15] have determined the rabbit ASGP-R in the nonionic detergent Brig 58 to have an approximate molecular weight of 234,000 daltons by sedimentation equilibrium. Based on a series of experiments using various denaturing conditions as well as covalent cross-linking, Kawasaki and Ashwell[16] have identified intermediates in the formation of the 250,000-dalton complex, which appears to be composed of A_2B_4.[15,16] In the presence of Ca^{++} and ligand and solubilized in Brig 58, the size of the complex (250,000 daltons) increases approximately twofold to 600,000 daltons.[15] This oligomeric aggregation occurs even in the presence of excess detergent and may have implications for the state of the receptor within biological membranes. Of interest are the observations of Steer et al.[17] on the size of the rabbit ASGP-R within the plasma membrane as assessed by radiation inactivation of ^{125}I-ASOR binding. By this technique, the functional receptor size is approximately 110,000 daltons. This could represent an (AB_2) composition, (AA, AB, or BB) dimers, or some combination of A/B subunits with an additional element of the plasma membrane (e.g., other proteins, glycolipids, etc.). As there is no current method for directly comparing the size of an integral membrane protein by sodium dodecyl sulfate-polyacrylamide gel electrophoresis or gel filtration with that obtained by radiation inactivation, clarification of the subunit composition must await further studies.

The size of the isolated rat ASGP-R in Brig 58 was found to be approximately 260,000 daltons by gel filtration.[15] In addition, the size of the functional receptor within rat liver plasma membranes was 104,000 daltons determined by radiation inac-

Table 1
SIZE OF ASIALOGLYCOPROTEIN RECEPTOR

Species	Mol wt in KD (minor species)	Method	Ref.
Rabbit	250	Gel filtration in Triton® -X-100	16
	234	Sedimentation equilibrium in Brig 58	15
	109	Radiation inactivation of plasma membrane	17
	48, 40	SDS-PAGE	16
	48, 40	SDS-PAGE	14
Rat	264	Gel filtration in Brig 58	15
	148	Radiation inactivtion of receptor in Triton® -X-100	17
	104	Radiation inactivation of plasma membrane	17
	47(54, 57)	SDS-PAGE	18
	54	SDS-PAGE	19
	52	SDS-PAGE following biosynthetic labeling	19
	42(51, 59)	SDS-PAGE following surface ^{125}I-iodination	20
	40(55, 65)	SDS-PAGE	21
	65, 55	SDS-PAGE following surface ^{125}I-iodination	21
	43(54, 64)	SDS-PAGE	22
	42, 48	SDS-PAGE following surface ^{125}I-iodination	23
Human	41	SDS-PAGE	24
	46	SDS-PAGE	25
	46	SDS-PAGE following biosynthetic labeling	25

tivation.[17] Both of these values are remarkably similar to that found for the rabbit receptor. In contrast, many observers report that only one major polypeptide species (40,000 to 54,000 daltons) results from sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the rat ASGP-R (Table 1).[18-22] In addition, all of the laboratories except Sawamura et al.[19] have identified two minor species of molecular weight approximately 55,000 and 62,000 daltons (Table 1). One-dimensional peptide maps suggest that all three species contain closely related, if not identical, polypeptides.[20,21]

In addition, both Schwartz et al.[21] and Harford et al.,[22] have generated monoclonal antibodies which recognize all three polypeptide species of rat ASGP-R. The presence of multiple forms of the receptor in isolated preparations raises questions as to the receptor organization within the plasma membrane. Warren and Doyle[20] identified all three receptor species on the isolated rat hepatocyte following ^{125}I iodination of surface proteins. However, since the reaction was performed at room temperature, endocytosis of radiolabeled surface membrane proteins may have occurred (see below, Section VI). In contrast, Schwartz et al.[21] demonstrated only the two higher molecular weight species on the cell surface following ^{125}I iodination at 4°C. Similarly, Fiete et al.[23] demonstrated two receptor species (42,000 and 48,000 daltons) on the rat hepatocyte surface. The exact relationship of the three species to one another remains unclear at present. Furthermore, Sawamura et al.[19] identified only a single species of receptor polypeptide by immunoprecipitation of biosynthetically labeled rat liver polysomes. This suggests that either post-translational processing and/or proteolytic cleavage may have resulted in the multiple species seen by the other investigators.

The human receptor appears to be a single polypeptide of molecular weight 41,000 daltons[24] or 46,000 daltons,[25] as judged from mobility on SDS-PAGE of the isolated receptor[24,25] or the receptor following biosynthetic labeling.[25]

The stoichiometry of binding between ligand and receptor is not clearly defined (see below, Section III). The principal difficulties with the current data are the actual size and composition of the functional binding unit (as discussed above) and the low specific binding activity of isolated receptor preparations. For example, the isolated receptor has a specific activity of approximately 40 to 80 ng of ligand (ASOR, molecular weight 40,000 daltons) per microgram receptor.[13-15,21,26] If one receptor molecule binds one ligand molecule and if the size of the receptor molecule is approximately 44,000 daltons (Table 1), then only 4 to 8% of these units are active. Similarly, if the size of the receptor binding unit is 250,000 daltons, then 25 to 50% are active. It is possible that the low activity is the result of irreversible denaturation, or proteolysis, during isolation. Alternatively, binding activity may depend upon the nature of the lipid environment. In this regard, Andersen et al.[15] have demonstrated different behavior of the isolated rat receptor in various nonionic detergents. Furthermore, Klausner et al.[27] quantified the reconstitution of the rabbit receptor into dipalmitoyl phosphatidylcholine liposomes. This association restored its ligand binding activity. The complex was unaffected by high salt, supporting a hydrophobic interaction and providing additional support for the receptor as an integral membrane protein.

Amino acid analysis has been performed on the rabbit receptor,[13] its A and B subunits,[16] the rat receptor,[19] and the human receptor.[24] The amino acid compositions of the A and B subunits of the rabbit receptor are essentially the same[16] and are nearly identical to those from rat[19] and human.[24]

ASGP-R from each species studied is a glycoprotein. The rabbit A subunit contains 9.9, 7.4, 10.7, and 10.2 mol of sialic acid, galactose, mannose, and *N*-acetylglucosamine per mole of polypeptide, respectively.[28] The B subunit contains 6.5, 5.6, 5.5, and 5.5 mol of these sugar residues per mole of polypeptide. The human receptor contains 6.1, 6.8, 8.5, and 11.7, mol of sugar per mole of polypeptide, respectively.[24]

Kawasaki and Ashwell[28] have determined the carbohydrate structure on the glycopeptides isolated from the rabbit receptor. The data are most consistent with subunit B containing 2 mol of the "complex" type oligosaccharides consisting of sialic acid, galactose, mannose, *N*-acetylglucosamine, and asparagine with a molar ratio of 3:3:2:5:1. Subunit A contains 3 mol of this same "complex" oligosaccharide and 1 mol of "polymannose" oligosaccharide containing mannose, *N*-acetylglucosamine, and asparagine at 8:2:1.[28] The terminal sugar residues of the "complex" oligosaccharide have been determined as sialic acid (alpha 2-6)-galactose-(beta 1-4)-*N*-acetylglucosamine.[29]

Recently, Schwartz and Rup[25] have identified N-linked oligosaccharides on the human receptor by biosynthetic labeling studies in the presence of tunicamycin, an inhibitor of the N-linked oligosaccharide precursor dolichol-phosphate, or by digestion of the biosynthetic intermediates with beta endoglucosaminidase H.

Thus, from the carbohydrate structure, it appears that the receptor itself is penultimate in galactose. Indeed, removal of sialic acid from the receptor within plasma membranes[30] or from the isolated solubilized receptor[13] resulted in complete loss of ligand binding activity. That this is a result of the exposure of terminal galactose residues on the receptor is suggesed by the observation that binding activity was restored by further treatment with beta galactosidase or galactose oxidase.[31] Furthermore, Paulson et al.[29] demonstrated that the neuraminidase-treated receptor was reactivated by incubation with CMP-*N*-acetylneuraminic acid in the presence of pure beta-D-galactoside alpha-2-6 sialyltransferase. Similarly, reactivation of the agalactoasialoreceptor was accomplished with UDP-galactose *N*-acetylglucosamine beta-1-4 galactosyl transferase, CMP-sialic acid beta D-galactoside alpha-2-6 sialyltransferase, UDP-galactose, and CMP-*N*-acetylneuraminic acid.[29]

Harford and Ashwell[32] suggest that the ASGP-R may be a transmembrane protein. Utilizing polyclonal antireceptor antibody, they demonstrated that distinct antigenic

determinants of the receptor reside on the cytoplasmic and extracytoplasmic surface of the hepatocyte plasma membrane. Less direct evidence in support of the ASGP-R as a transmembrane protein is provided by analogy to the closely related avian hepatic receptor which is *N*-acetylglucosamine specific.[33] Drickamer[34] has provided the entire amino acid sequence of the receptor. Recently, Drickamer and Mamon[35] demonstrated a phosphorylation site on serine$_7$ followed by 25 uncharged hydrophobic amino acid residues and thereafter the location of an oligosaccharide on residue 67. This sequence suggests that the avian hepatic *N*-acetylglucosamine receptor has a transmembrane orientation. Additional evidence related to the orientation of the ASGP-R in the plasma membrane is provided by Nakada et al.[36] Using isolated polysomes and biosynthetic labeling, they suggested that the carbohydrate portion of the receptor is better protected from external protease digestion than is the amino acid portion. However, the specificity of the antibody used for the receptor identification as well as for the receptor polypeptide's association with polysomes may not be complete.

Schwartz and Rup[25] have examined the biosynthesis of the human ASGP-R in a human hepatoma cell line using [^{35}S]-amino acid and monospecific anti-human receptor antibody. The receptor is initially synthesized as a 35,000-dalton precursor which is converted with a $t_{1/2}$ of 45 to 60 min at 37°C to the 46,000-dalton mature species by the addition of N-linked oligosaccharide in the Golgi. The mature receptor then rapidly appears at the cell surface. The human receptor has a mean lifetime ($t_{1/2}$/ln2) of approximately 30 hr in these cells. Previously, Tanabe et al.[18] determined a mean lifetime of approximately 125 hr for the affinity purified rat ASGP-R from rats administered [^{3}H]-leucine. In addition, Warren and Doyle[20] found a mean lifetime of approximately 30 hr for the receptor in isolated hepatocytes compared to 120 hr for the typical rat hepatocyte surface protein.

Thus, the ASGP-R is an integral membrane glycoprotein. It requires detergent for removal for the membrane. The isolated ASGP-R retains its ligand binding activity in the presence of detergents. When isolated it is composed of variable numbers of subunits, albeit with some species differences. The subunit size is approximately 50,000 daltons. Depending upon the species, there may be 2 to 3 N-linked oligosaccharides per subunit. The ASGP-R is synthesized on polyribosomes and is post-translationally modified similar to that of most membrane glycoproteins.

III. STRUCTURAL REQUIREMENTS FOR LIGAND BINDING

The physiological functions associated with the ASGP-R are dependent upon its ability to recognize ligands of the appropriate class, i.e., structure. This stringency thus defines the ligand specificity. The structural requirements which result in binding of the ligand to the ASGP-R were initially addressed using chemically and enzymatically modified derivatives of naturally occurring glycoproteins. Binding studies in vitro revealed a high degree of specificity for those glycoproteins in which the penultimate galactose residue had been made terminal (see Ashwell and Morell[1] and Kawasaki and Ashwell[16]).

An early study by Stockert et al.[31] examined competition of ^{125}I-ASOR binding to the isolated rabbit receptor. While inhibition with D-galactose was 50% effective [I_{50}]* at 28 m*M*, fetuin or ceruloplasmin which terminated in D-galactose were more than 4000 times more effective. Furthermore, desialo-bovine submaxillary mucin (terminating in *N*-acetylgalactosamine residues) was yet more effective by an additional 100-fold. The first systematic evaluation of the structural requirements of the binding of ligand to the isolated receptor was performed by Sarkar and colleagues.[37] Radiolabeled

* [I_{50}] is the concentration sufficient to yield 50% inhibition. When the standard ligand is used at "tracer" concentrations, the resultant [I_{50}] of a test substance is equal to the K_d.

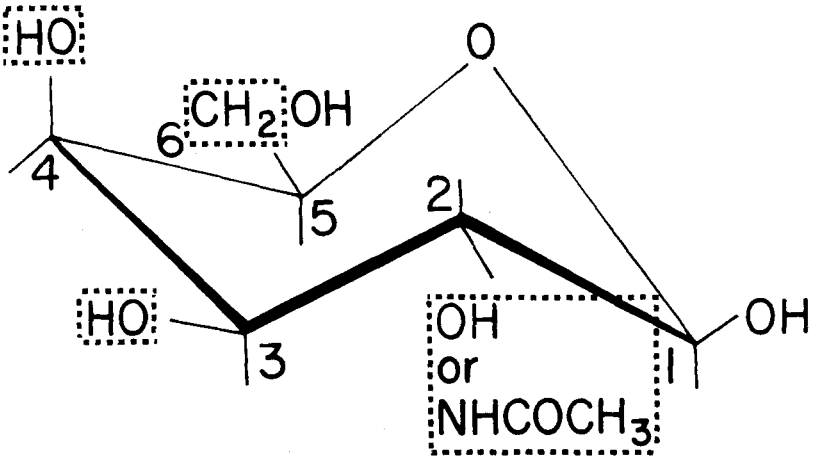

FIGURE 1. Structure of D-galactose and D-*N*-acetylgalactosamine. The essential groups which govern the interaction with the ASGP-R are outlined in dashed boxes: 2-OH or 2-$NHCOCH_3$ equatorial, 3-OH equatorial, 4-OH axial and 6-CH_2OH. The nature of the aglycon adds additional constraints to ligand recognition.

OG 20% 2X, an ovarian cyst precursor blood group substance (with blood group I and i activity), containing many terminal nonreducing D-galactose residues in beta linkage, was used in a competitive assay for binding to isolated purified rabbit liver receptor immobilized on Sepharose beads. At optimal pH (7.8) and calcium concentrations, the inhibitory effects of various monosaccharides, glycosides, oligosaccharides, polysaccharides, glycoproteins, and blood group substances were examined. Methyl-alpha-D-*N*-acetyl galactose was the best inhibitor of the monosaccharides studied, about one order of magnitude better than D-*N*-acetyl galactose or D-galactose and approximately the same as the best disaccharide examined, D-galactose beta 1-3-D-*N*-acetylglucosamine. However, the inhibitory effects of all mono- and disaccharides examined fell within 1.5 orders of magnitude, less than that generally expected for a simple competitive process involving both homogeneous acceptor and competitor species. Nonetheless, ASOR, which is terminal D-galactose-(beta 1-4)-*N*-acetylglucosamine, was more than 2000 times a better competitor than any mono- or disaccharide. A wide variety of naturally occurring polysaccharides and blood group substances were found to be as or more effective than ASOR. Most of these compounds have terminal nonreducing alpha-linked *N*-acetyl galactosamine or terminal nonreducing beta-linked D-galactose residues. Based on these studies, the presence of terminal *N*-acetylgalactosamine or D-galactose with an axial hydroxyl on C4 was necessary for binding (Figure 1).

These data are consistent with earlier studies by Kawasaki and Ashwell[16] and Stockert et al.[6] who demonstrated that purified rabbit ASGP-R agglutinates human A erythrocytes (which terminate in *N*-acetylgalactosamine) more strongly than either B or O erythrocytes. *N*-Acetyl-D-galactose was a better inhibitor of agglutination than D-galactose.

Another approach to the problem of specificity of ligand binding has been taken by Lee and associates, who have made use of synthetically derived neoglycoproteins.[38] Neoglycoproteins were prepared by modifying bovine serum albumin (BSA) via amidation or reductive alkylation with thioglycosides of mono- or disaccharides. Initial studies[39,40] using glycosyl-BSA derivatives as competitors for ^{125}I-ASOR binding to the isolated rabbit receptor demonstrated that glucose-BSA derivatives effectively interacted with the receptor as well as galactose-BSA derivatives. In addition, *N*-acetyl galactose-BSA was much more effective as a competitor than either of these compounds,

while *N*-acetyl glucose derivatives as well as mannose derivatives were without effect. However, more recent experiments with various sugar-protein linkages have demonstrated that an axial hydroxyl on carbon 4 of galactose is essential for binding, while the C-6 position is less stringent, but cannot have a negatively charged group. Additional determinants of ligand recognition include 2-OH (equatorial) and 3-OH (equatorial)[40,41] (Figure 1). The variable efficacy of the glucose-BSA conjugates may suggest that the nature of the sugar to protein linkage may improve the otherwise weak binding of glucose, perhaps via alterations in charge.[41] This hypothesis may explain earlier studies by Stowell and Lee,[39] in which D-galactosyl- and D-glucosyl-neoglycoproteins obtained via stable amidine linkages were capable of binding to the rabbit hepatic asialoglycoprotein receptor. In addition, using these D-galactosyl-BSA and D-glucosyl-BSA ligands, they were able to affinity purify the identical hepatic ASGP-R. Their criteria included identity on sodium dodecyl sulfate polyacrylamide gels, carbohydrate composition, amino acid composition, binding affinities, and cross reactivity of antibodies.[39]

Most recently, Lee et al.[42] have extended their studies using a variety of glycosides as inhibitors of ^{125}I-ASOR binding to the isolated rabbit receptor to probe the role of the C-6 region of the sugar-ligand. Sugars possessing a C-6 methylene group were tenfold better inhibitors. In addition, a variety of aglycones "strengthened the binding of galactose to the receptor" (i.e., reduced the $[I]_{50}$). With these aglycones there was a substantial increase in the binding of glucose to the receptor as well, especially for the two gucosides which contained the aglycone structure: $SCH_2C(=NH)NH-$. The aglycones possessing a chain length of three atoms or longer increased the binding of galactose (or glucose). Aglycones had little or no effect on mannose or *N*-acetylglucosamine.

Therefore, from the studies of Stowell and Lee,[39] Stowell et al.,[40] Sarkar et al.,[37] Lee,[41] and Lee et al.,[42] five groups on the galactopyranoside or *N*-acetylgalactopyranoside have been shown to participate in ligand-receptor interactions. As seen in Figure 1, these are the aglycone, 2-OH or 2-$NHCOCH_3$, 3-OH, 4-OH, and 6-CH_2-.

Most naturally occurring glycoproteins contain branched chain, or multiantennary, oligosaccharides. In addition, many such as orosomucoid contain substantial heterogeneity at each glycosylation site.[43] Therefore, it was not unexpected to hypothesize that the number of terminal galactose or *N*-acetylgalactosamine residues as well as their density or "clustering" may be important for ligand recognition. Studies using model neoglycoproteins with various ratios of galactose to polypeptide (e.g., albumin) have demonstrated a direct correlation with the overall galactose density.[44] More recently, galactose-BSA derivatives were prepared via reductive amination.[45] These ligands have a variable distance separating the galactose residue from the polypeptide backbone. The potency of these ligands was shown to improve with increased sugar-protein distance, suggesting that the binding of galactose moieties to the receptor may require a certain degree of proximity between sugar groups; thus, a "cluster" phenomenon (Figure 2).

In order to address this "cluster" effect, Baenziger and Maynard[24] examined a variety of naturally occurring glycopeptides for their abiliy to inhibit ^{125}I-ASOR binding to the isolated human receptor. They demonstrated a 30-fold lower k_i for triantennary complex oligosaccharides compared to biantennary complex oligosaccharides, all of which terminated in galactose.

A major limitation of these studies involves the use of isolated receptor preparations. As discussed above (Section II), standard receptor preparations have lost substantial ligand binding activity during isolation. Thus, it is not certain whether that ligand binding activity which remains is a representative subpopulation of total ligand binding activity or whether all ligand binding sites are functionally identical.

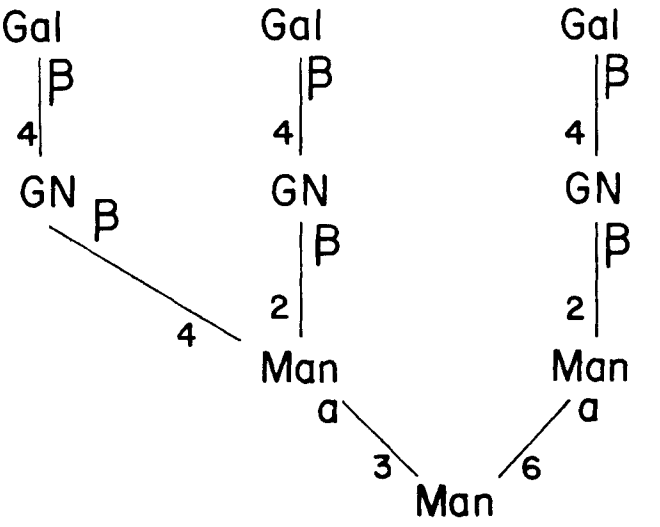

FIGURE 2. Structure of galactose-terminal "complex" oligosaccharide commonly found in serum galactose-terminal glycoprotein ligands: Gal = galactose, GN = *N*-acetylglucosamine, Man = mannose.

In order to examine this potential difficulty as well as the ASGP-R within its native membrane environment, Baenziger and Fiete[46] examined the inhibitory activity of various glycopeptides both on binding of ^{125}I-ASOR to isolated rat hepatocytes as well as their subsequent uptake. Their data demonstrated substantial differences of ligand receptor binding when studied with the isolated rat liver receptor, compared to the receptor on isolated rat hepatocytes. For example, an 800-fold difference in inhibition potency of two glycopeptides (IgA 1/GPI and agalacto IgA 1/GPI) was found when examined in an inhibition assay using isolated receptor and ^{125}I-ASOR. IgA 1/GPI contains four terminal galactose residues and one terminal *N*-acetylgalactosamine residue while agalacto IgA 1/GPI contains four terminal *N*-acetylgalactosamine residues only and in positions similar to the terminal galactose residues in the parent compound. The K_d for binding determined at 4°C in isolated hepatocytes varied by 35-fold, whereas the K_d for uptake (K_{uptake}, a measure of binding coupled with endocytosis) varied less than 2-fold. This suggested substantial differences in structural requirements for ligand binding compared to uptake.

More recently, Connolly et al.[47] have confirmed the inability of the isolated receptor, in this case from rabbit liver, to effectively discriminate many structural features of their synthetic mono-, bi-, and triantennary galactose- or lactose-terminated cluster glycosides. However, they found substantial differences in binding of these cluster glycosides to isolated rabbit hepatocytes in culture. The concentration of triantennary (tris-glycosides) and biantennary (bis-glycosides) ligands were 10- and 100-fold lower than that of monoantennary (monoglycosides) required for 50% inhibition of ^{125}I-ASOR binding. These differences underscore the requirements for galactose density as well as spacing.

The uptake in rat hepatocytes of neoglycoproteins, including both monosaccharide (galactose and glucose) derivates as well as BSA derivatives containing trisgalactosides or trisglucosides, was examined by Kawaguchi et al.[48] They extended the earlier studies of Stowell et al.[40] by demonstrating that amidino-glucosyl-BSA not only binds avidly to the receptor, but is taken up as effectively as galactose-BSA compounds. However, other types of glucosyl-BSA linkages did not yield an active ligand. In addition, the

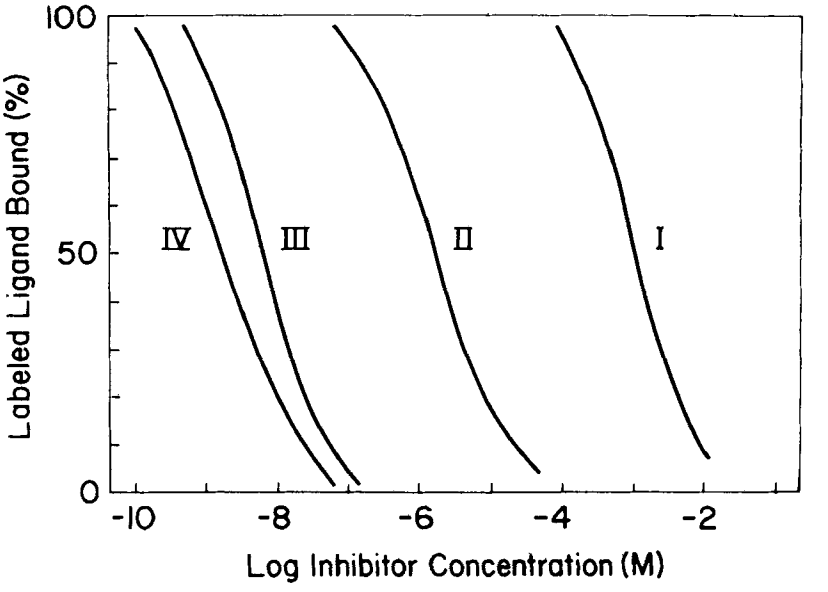

FIGURE 3. Inhibition of binding of ^{125}I-Tyr-asialotriantennary glycopeptide to isolated rabbit hepatocytes at 2°C. Unlabeled ligands were incubated with freshly isolated hepatocytes at the indicated concentrations. The monoantennary ligand (I) used was Galβ(1,4)GlcNacβ(1,6)Man. Asialobiantennary glycopeptide from human fibrinogen was used as the biantennary ligand (II). The triantennary ligand (III) used was the asialotriantennary glycopeptide from human α-1-protease inhibitor. The tetrantennary ligand (IV) was a synthetic oligosaccharide terminating in galactose. Adapted from Lee et al.[49]

trisgalactoside of BSA was rapidly taken up, while the trisglucoside-BSA was very poorly recognized, if at all.

In order to minimize the potential difficulties associated with natural glycopeptide heterogeneity as well as to examine the ligand binding site in a native environment within the plasma membrane, Lee and colleagues[49] have further examined the features of ligand-receptor binding in isolated rabbit hepatocytes. Oligosaccharides with precisely defined structure were used as inhibitors of binding of either ^{125}I-ASOR or ^{125}I-tyrosine-asialotriantennary glycopeptide derived from alpha 1 protease inhibitor. As seen in Figure 3, there was a dramatic hierarchy of inhibitory potencies: tetra >, tri ⩾, bi ⩾, monoantennary oligosaccharides displayed $[I]_{50}$ of about 10^{-9}, 5×10^{-9}, 10^{-6}, and 10^{-3} *M*, respectively. Galactose, alone, had an $[I]_{50}$ similar to that found for the monoantennary compounds. Similar experiments with various tri- and tetra-antennary oligosaccharides demonstrated slight differences in inhibitory potency depending upon the position of the attachment of the beta-*N*-acetyllactosamine chains.

Thus, these studies suggest that the number of galactose or *N*-acetylgalactosamine residues per cluster, their branching pattern, and the distances between galactose residues are major determinants in the recognition of ligand by receptor.

A. Interaction of Ca^{++} with ASGP-R

There exists an absolute requirement for Ca^{++} in the binding of a ligand to the ASGP-R. This was first demonstrated by Pricer and Ashwell[30] using rat liver plasma membranes. Optimal Ca^{++} concentration for binding to the plasma membrane was approximately 2 m*M*.[50] In isolated rat hepatocytes, Tolleshaug and Berg[51] and Weigel[52] have further defined this absolute Ca^{++} requirement (optimal concentration approximately 0.1 m*M*). Dissociation of prebound ligand occurred with $t_{1/2}$ approximately 0.5 min in

the presence of EGTA. Thus, the requirement for Ca^{++} provides a rapid and sensitive means for controlling association/dissociation of ligand and ASGP-R (see below, Sections V and VI).

Recently, Blomhoff et al.[53] demonstrated that the binding of $^{45}Ca^{++}$ to isolated rabbit ASGP-R required the presence of ligand. The isolated receptor complex, which was assumed to be approximately 250,000 daltons, bound 1 ligand molecule and 3 to 4 Ca^{++} ions. Scatchard and Hill analyses were consistent with positive cooperativity. Since the 250,000-dalton complex of the rabbit ASGP-R is probably a complex of 4 to 6 subunits, based on subunit size,[16] it is likely that there was one Ca^{++} bound per single subunit or per dimer. In contrast, Andersen et al.[15] found 48 $^{45}Ca^{++}$ ions bound per isolated rabbit ASGP-R complex (600,000 daltons in their studies). Scatchard analysis of these data revealed a high affinity component of K_d = 0.35 m*M* and a second lower affinity component. These studies were performed in the presence of Brig 58, whereas those of Blomhoff et al.,[53] were performed in the presence of Triton® -X-100. In addition, Andersen et al.,[15] separated bound and free $^{45}Ca^{++}$ by ultrafiltration, whereas Blomhoff et al.[53] used equilibrium dialysis. Despite these dissimilarities, it is not readily apparent why such differences in the amount and kinetics of Ca^{++} binding exist.

Blumenthal et al.,[54] demonstrated a direct effect of Ca^{++} on the orientation of the isolated rabbit ASGP-R in cholesterol black lipid membranes. Addition of the ASGP-R to the cis side of the lipid membrane generated an asymmetric increase in transmembrane conductance. This suggested that the protein was (partially) inserted in the membrane and increased ion permeability. Following the addition of Ca^{++} alone to the cis side of such ASGP-R membrane preparations, the conductance became symmetrical, consistent with a change in the position of the ASGP-R with respect to the black lipid membrane. Similar observations have been made upon addition of ligand alone. The simplest interpretation of these results is that ligand alone or Ca^{++} alone is able to initiate movement of (part of) the ASGP-R to the opposite side of the membrane.

B. Kinetic Analysis of Binding of ASOR to the ASGP-R

As discussed above (Section II), the receptor isolated from each mammalian source is extensively inactivated during preparation or represents a large oligomer with only a limited number of binding sites, despite the presence of identical subunits. The rabbit receptor has been best characterized.[13,16,28] The rabbit receptor consists predominantly of two polypeptides, an A and a B chain with apparent molecular weights 48,000 and 40,000 daltons, respectively, as determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Both polypeptides contain complex oligosaccharides.[28] Since the Ca^{++}-receptor complex is inactive as well as insoluble in the absence of detergents, delineation of the binding properties of the receptor requires the presence of aqueous solutions of detergents. Hudgin et al.[13] originally described the characteristics of binding of ASOR to the isolated rabbit ASGP-R in the presence of Triton® -X-100. Receptor-bound ASOR was separated from free ligand by differential precipitation in ammonium sulfate. Kinetic studies of the formation of the ligand-receptor complex demonstrated a $t_{1/2}$ of association of less than 2 min. On the other hand, dissociation was neither rapid (nondetectable by 60 min in the absence of added free ligand and less than 25% dissociated by 60 min in the presence of excess free ligand) nor complete at room temperature. The stability of the ligand-receptor complex was found to rapidly decrease at temperatures above 25°C.

These studies have been extended more recently by Connolly et al.[14] who determined the binding constants at 0°C for ^{125}I-ASOR and the isolated rabbit receptor by both steady state and kinetic techniques. They demonstrated two classes of binding sites, present in approximately equal numbers, with apparent equilibrium dissociation constants (K_{app}) of 0.87 and 1100 n*M*. Scatchard plot analysis revealed similar dissociation

constants. The forward binding kinetics revealed an apparent rate constant of $K_1 = 6.8 \times 10^6\ M^{-1}\ min^{-1}$ and $K_{-1} = 1.5 \times 10^{-2}\ min^{-1}$, thus yielding $K_{app} = 2.1$ n*M*. The presence of a 500-fold molar excess of free ASOR did not cause a striking dissociation of the ligand-receptor complex. Other neoglycoprotein derivatives (e.g., galactose-BSA) were also unable to significantly displace bound ligand from receptor. In contrast, there was a very rapid dissociation of ligand from receptor in the presence of methyl beta D-galactose. (These data are similar to those obtained with receptor on the cell surface [see below, Section V]).

Thus, the isolated ASGP-R in detergent solution displays rapid kinetics of ligand binding, while dissociation is very slow at best, even in the presence of substantial ligand excess. However, simple sugars at high concentration could promote rapid dissociation of ligand from ASGP-R.

IV. RECEPTOR DISTRIBUTION

The ASGP-R is localized exclusively to the parenchymal cells of the liver (hepatocytes). Following i.v. administration of ^{125}I-ASOR to rats, Hubbard et al.,[55,56] demonstrated by electron microscopic autoradiography that galactose-terminated glycoprotein ligands were bound and internalized only by hepatocytes. Similarly, Tolleshaug et al.[57] demonstrated biochemically that asialofetuin was only taken up by liver parenchymal cells. The kinetics of hepatic clearance of ^{125}I-ASOR in the rat in vivo have recently been quantified by Partridge et al.[57a] with a portal vein injection technique. They determined a maximal clearance rate of 320 μg/min/g for ASOR and 240 μg/min/g for asialofetuin. Subcellular fractionation of whole rat liver demonstrated substantial receptor activity in smooth microsomes and Golgi, in addition to the plasma membrane fraction.[26] Purified receptor isolated from each of these fractions was shown to possess similar ligand binding specificities as well as to cross react to the same degree with goat antireceptor antibody.

Studies with the isolated perfused rat liver, which maintains physiological cellular associations, have demonstrated that the galactose-terminal ligands preferentially bind to coated pit areas of the plasma membrane. In liver maintained at 4°C to prevent ligand internalization, Wall and Hubbard[58] used ligands coupled to horseradish peroxidase or ferritin for electron microscopic visualization of ligand binding. They found binding sites present over much of the sinusoidal cell surface, but concentrated more than 70-fold in clathrin coated pits. Similar findings have been reported in rats in vivo by Stockert et al.[59] using ligand coupled to horseradish peroxidase or tyrosinase. In addition, these investigators also identified specific reaction products along the lateral cell surface. Of interest are the observations by Hardonk and Scholtens[60,61] of a zonal heterogeneity of histochemical ligand binding within the rat liver with increasing activity in the area surrounding the central vein. Distribution of the receptor on the rat hepatocyte surface has been performed on cells fixed in vivo with gluteraldehyde.[62] Using antireceptor antibody coupled to ferritin, approximately 90% of the receptors were localized to the sinusoidal surface with about 10% on the lateral surface and 1% on the bile canalicular face. The receptors on the sinusoidal surface have a near random distribution. No immunolabeling was associated with Kupffer cells or other sinusoidal-lining cells.

Recently, Geuze et al.[63,64] have described the localization of the ASGP-R in rat liver in vivo using antireceptor antibody and quantitative immunocytoelectron microscopy with colloidal gold (Figure 4). Localization was confined to parenchymal cells (hepatocytes); Kupffer cells were negative. About 35% of the total cell receptors were identified at the plasma membrane surface. Of these, approximately 85% were at the sinusoidal plasma membrane while the remaining 15% were localized to the lateral cell

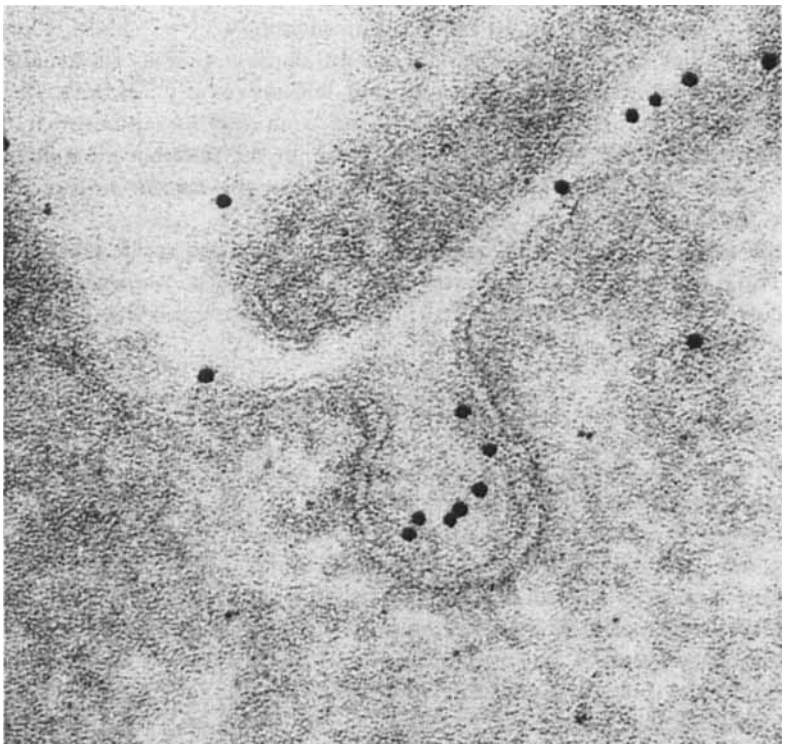

FIGURE 4. Immunocytochemical electron micrograph of coated pit containing ASGP-R. A thin cryosection of perfusion-fixed rat liver was labeled with affinity purified anti-rat ASGP-R antibody and 8 nm colloidal gold-protein A. The section shows an hepatocyte coated pit containing abundant ASGP-R. Additional receptor is seen along the plasma membrane surface. (H. J. Geuze and A. L. Schwartz).

surface and to the bile canalicular membrane. Receptors were concentrated within coated pit areas as had been described earlier by Wall and Hubbard.[58]

Isolated hepatocytes in suspension or monolayer culture provide an excellent system for more precise biochemical studies of receptor function. However, as pointed out by Zeitlin and Hubbard,[65] the preparation of isolated cells often results in a loss of recognizable differentiated membrane domains (e.g., sinusoidal, lateral, bile canalicular). Not surprisingly, Weigel[66] reported a diffuse receptor distribution over the entire surface of isolated hepatocytes. Using electron micrographic autoradiography, Zeitlin and Hubbard[65] also demonstrated a random distribution of ligand binding sites over the entire cell surface. However, the horseradish peroxidase reaction product of the ligand-horseradish peroxidase complex was concentrated in coated pit areas of the cell surface following ligand binding at 4°C. Thus, both ligand binding sites as well as the receptor itself are localized predominantly to the sinusoidal surface of the plasma membrane where they are concentrated in coated pits.

Although isolated hepatocytes provide a convenient system in which to examine the fate of ligand and receptors subsequent to binding, the method of isolation (see Carlsen et al.[67]) has resulted in a wide range of biochemical parameters related to the ASGP-R. For example, the number of functional receptors on the isolated rat hepatocyte surface as assessed by saturation binding of ligand (generally ^{125}I-ASOR) has been reported as 30,000 to 500,000 per cell.[46,52,65,68-71] Many of these variables have recently been summarized by Zeitlin and Hubbard[65] who pointed out variability in receptor

number that could be attributed to the use of different lots of collagenase in the cell preparation. Weigel and Oka[72] have provided evidence for the existence of a pool of cryptic (not simply occupied) surface receptors, which are exposed following treatment with EDTA. In addition, they[52,72] have demonstrated a reversible modulation of total surface receptors by alterations in temperature. The number of cell surface receptors increases in proportion to temperature above 17°C. The mechanisms responsible for these alterations are not presently understood.

Despite these striking differences in the number of cell surface receptors and their availability, there is general agreement that there exists a substantial intracellular pool of functional receptor, at least in rat hepatocytes. By detergent solubilization, Steer and Ashwell[69] initially demonstrated the vast majority of ASGP-R in isolated rat hepatocytes was intracellular. Baenziger and Feite[46] also identified such a pool following sonic disruption. Subsequently, Weigel and Oka[73] and Weigel et al.[74] have provided additional evidence for a large intracellular pool of receptor in rat hepatocytes determined following digitonin permeabilization. These studies are consistent with the earlier cell fractionation studies of Pricer and Ashwell[26] and Tanabe et al[18] which indirectly suggested a substantial intracellular pool of receptors. Additional studies of Bridges et al.[75] and Stockert et al.[76,77] support the presence of a large intracellular pool of receptor in rat hepatocytes (see below, Section VI). As discussed below, there is evidence that not all ASGP-R function identically. An independent assessment of the large intracellular pool of ASGP-R in rat liver was performed by Geuze et al.[64] using quantitative immunocytochemistry with antireceptor antibody. Their study revealed approximately 20% of the intracelluar receptor in the RER, approximately 30% in the Golgi, and approximately 50% in the smooth endoplasmic reticulum and endocytic vesicles (i.e., CURL, see below, Section VI). The Golgi distribution of the ASGP-R is nearly uniform across the entire stack, although the transcisternae may contain somewhat higher amounts.[78]

Although the ASGP-R is confined to parenchymal cells of the liver, interest in the possible existence of other galactose-specific receptors stemmed from studies which examined the removal of native or desialylated erythrocytes and lymphocytes from the circulation. Kolb et al.[79] demonstrated tight binding of sialidase-treated rat or mouse erythrocytes to isolated hepatocytes. Binding was calcium dependent and inhibited by galactose, fucose, or *N*-acetylgalactosamine, but not by mannose, *N*-acetylglucosamine, or glucose. Binding to contaminating Kupffer cells was also observed.[80] In addition, Muller et al.[81] have demonstrated a role for galactose in the binding of desialylated erythrocytes to macrophages. Recently, Kolb-Bachofen et al.[82] provided evidence for the presence of a galactose/*N*-acetylgalactosamine-specific receptor on the Kupffer cell surface by quantitative binding of colloidal gold particles adsorbed with asialofetuin or galactose-terminated bovine serum albumin. The binding was calcium dependent and was specifically inhibited by *N*-acetylgalactosamine, but not by mannose. However, gold adsorbed with BSA alone bound about 50% as well as gold-BSA-galactose.[82] Whereas *N*-acetylgalactosamine at 25 m*M* inhibited 97% of gold-asialofetuin endocytosis, fucose, mannose, and glucose inhibited 50, 25, and 25%, respectively, at 25 m*M*.[83] Since, as discussed above, the hepatic ASGP-R is not associated with Kupffer cells, nor do galactose-terminal plasma glycoproteins associate with or accumulate within Kupffer cells,[55,56] it is possible that the process in Kupffer cells may be confined to "particles" (e.g., erythrocytes) possessing galactose-terminal residues. Future studies will be necessary to more critically define this recognition phenomenon.

Thus, the hepatic ASGP-R is localized exclusively to the hepatocyte which contains 100,000 to 500,000 receptors/cell. The receptors at the cell surface are randomly distributed over the sinusoidal surface with enrichment at coated pits. Within the cell, the majority of ASGP-R are associated with the endocytic organelles and Golgi.

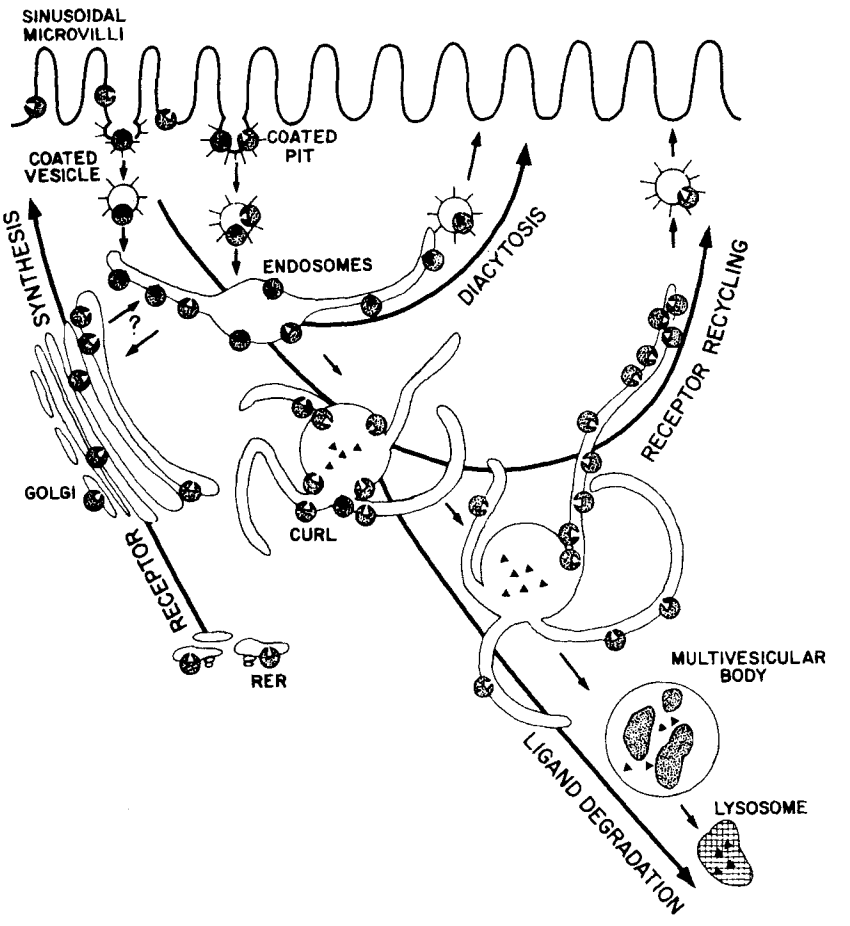

FIGURE 5. Pathway of receptor-mediated endocytosis for ligand and ASGP-R. Receptor is randomly distributed on the hepatocyte sinusoidal plasma membrane. Ligand-receptor complexes enter the cell in coated pits/coated vesicles. Upon loosing their coats, these vesicles fuse to yield smooth surfaced vesicle/tubules (endosomes). Ligand is continuously delivered to the central vesicle by movement through the thin tubules as these endocytic structures increase in size. Some ligand in association with receptor is exocytosed from this early compartment, perhaps via a coated intermediate (diacytosis). The bulk of ligand dissociates from receptor within the vesicular portion of compartment of uncoupling receptor and ligand (CURL). The unoccupied receptor molecules fill the tubular structures on route back to the cell surface, perhaps via a coated intermediate (receptor recycling), while the ligand molecules within the vesicular portion enter multivesicular bodies prior to the lysosomes (ligand degradation). Constitutive recycling of unoccupied receptors is described, although the exact pathway is not certain. The receptor is synthesized in the rough endoplasmic reticulum (RER), transported to the Golgi, where oligosaccharide modification occurs, and rapidly arrives at the plasma membrane surface. It is not presently clear whether recycling receptor interacts with the Golgi elements.

V. FATE OF THE GALACTOSE TERMINAL LIGAND

Galactose-terminal glycoprotein ligands avidly bind to the ASGP-R on rat hepatocytes prior to their internalization, intracellular routing to lysosomes, and degradation. Binding of ^{125}I-ASOR to isolated rat hepatocytes is rapid, [$K_{on} > 1.8 \times 10^4\ M^{-1}\ sec^{-1}$],[52] while dissociation of prebound ligand is slow [$K_{off} < 0.9 \times 10^{-5}\ sec^{-1}$].[52] Equilibrium binding studies demonstrated a K_d approximately $2 \times 10^9\ M^{-1}$.[46,52,69,70] Binding data and Scatchard analysis suggest a single high affinity class of binding sites.

The human hepatoma cell line Hep G2 contains approximately 150,000 to 200,000 surface ligand binding sites per cell.[84] These receptors represent a homogeneous popu-

lation of high affinity sites with K_d for ^{125}I-ASOR approximately 7×10^{-9} *M*. Similar to rat hepatocytes, binding is rapid with K_{on} approximately 1.6×10^4 M^{-1} sec.$^{-1}$.[84]

Once bound at the cell surface in coated pits, the ligand is rapidly internalized into coated vesicles and thereafter into endocytic vesicles and tubules (Figure 5). Hubbard and Stukenbrok[56] followed the kinetics of ^{125}I-ASOR uptake in rat liver in vivo by electron microscopic autoradiography. By 1 to 2 min at 37°C, 40% of the cell-associated ligand had left the plasma membrane and was predominantly confined to the peripheral cytoplasmic area. Using electron-dense markers (ASOR-horseradish peroxidase or galactose-ferritin), Wall et al.[85] extended these observations to demonstrate the initial (within 30 sec) appearance of ligand in coated vesicles (approximately 0.1 μm diameter) and larger smooth surface vesicles and tubules in the peripheral cytoplasm. The ligand was initially closely opposed to the membranes of the coated vesicles, but it was found throughout the lumen of larger vesicles. They suggested that this may represent dissociation of ligand from receptor (see below, Section VI). At 5 min, the tracers began to appear in the lysosome-Golgi region within interconnecting tubules. Following 15 min, virtually all ligand was in the Golgi-lysosome region, mostly within secondary lysosomes in which degradation of ligand occurred.[86-88] A similar pathway for ligand movement has been described by electron microscopy in isolated hepatocytes,[65] hepatocytes in culture,[65,89] and hepatoma cells in culture[90] (Figure 5).

Movement of ligand from the cell surface to the lysosome has been dissected biochemically in rat hepatocytes. Tolleshaug et al.[57] demonstrated rapid uptake (binding and internalization) of ^{125}I-asialofetuin with the subsequent appearance of degradation products in the medium after a lag of approximately 20 min at 37°C. Steer and Ashwell[69] similarly demonstrated a lag of 20 to 30 min in the appearance of degradation products of ^{125}I-ASOR.

The internalization event can be described as a first-order process; the fractional extent of internalization of prebound ligand is constant over a wide range of receptor occupancy. In the rat hepatocyte, Tolleshaug et al.[91] reported a rate constant ($\ln 2/t_{1/2}$) of 0.18 min^{-1} for internalization of ^{125}I-ASOR at 30°C. Weigel and Oka[71] and Bridges et al.[75] found rate constants of 0.12 to 0.23 and 0.2 min^{-1}, respectively, at 37°C. Schwartz et al.,[92] in human hepatoma cells, reported a rate constant for internalization of 0.46 min^{-1}. Internalization, but not ligand binding, is an energy-dependent process which can be inhibited by metabolic energy inhibitors[92,93] or by low temperature (e.g., 0°C).

A. Diacytosis

Endocytosis of galactose-terminal ligands of the ASGP-R does not lead to unidirectional uptake and delivery of ligand to lysosomes (Figure 5). Studies from the laboratory of Regoeczi have recently delineated an ASGP-R-dependent pathway which results in exocytosis of a portion of the internalized ligand. A substantial fraction (up to 75%) of human asialotransferrin type 3, a ligand both for the ASGP-R as well as the hepatic transferrin receptor,[94] is released back into the media following uptake by rat hepatocytes.[95] This released ligand was identical in size to asialotransferrin prior to exposure to the cells. The extent of release was dependent on the initial ligand concentration with a greater fraction of exocytosed material found at lower ligand concentrations. Regoeczi et al.[95] demonstrated that although this ligand may bind to transferrin receptor on hepatocytes, at least the majority of the initial binding of the asialotransferrin is specific for the ASGP-R. Earlier studies demonstrated that asialotransferrin was of lower affinity in recognition of the ASGP-R than many other desialylated plasma proteins.[96] The kinetics of in vivo clearance of ^{125}I-asialotransferrin suggest that competitors released this ligand predominantly by preventing recapture of the exocytosed free ligand. Transferrin itself diacytoses through the cell during receptor-me-

diated endocytosis for the delivery of iron,[97] including the liver cells.[98] Thus, it is possible that asialotransferrin enters the cells via the ASGP-R, dissociates from this receptor, and then rebinds to the transferrin receptor for its return to the cell surface. However, this may explain only some of these observations, since a significant fraction (approximately 25%) of the ^{125}I-asialotransferrin is resialylated during its diacytosis in rat liver in vivo.[99] This process is slow, requiring hours. The nature of this diacytotic pathway remains unclear at present.

Other galactose-terminal ligands have recently been shown to exocytose from liver cells. Connolly et al.[47] demonstrated that a synthetic cluster tris-glycoside terminating in galactose was rapidly ($t_{1/2} < 5$ min) and extensively (> 95%) exocytosed by rabbit hepatocytes following binding and internalization (as judged by resistance to EDTA). This process specifically involved the ASGP-R as demonstrated by competition studies and use of antireceptor antibodies. This synthetic cluster glycoside was spared lysosomal degradation and returned to the media intact. In studies of single-cycle kinetics of exocytosis of ^{125}I-ASOR in hepatoma cells, Simmons and Schwartz,[99a] found 28% of the internalized ^{125}I-ASOR in the medium with $t_{1/2} \sim 80$ min. In the presence of *N*-acetylgalactosamine, 45% of the ^{125}I-ASOR was exocytosed ($t_{1/2} \sim 20$ min). In rat hepatocytes, Townsend et al.[100] demonstrated exocytosis of 6 and 17% of ^{125}I-ASOR endocytosed over 20 and 5 min at 37°C, respectively. On the other hand, the alpha 1 antitrypsin glycopeptide demonstrated 40 and 60% exocytosis under similar conditions. Thus, the nature of the ligand, and perhaps its affinity for the ASGP-R, governs targeting of the ligand to the lysosomes vs. its exocytosis from the hepatocyte. The details of this process are yet to be fully established.

B. Modulation of Ligand Movement

The availability of a group of pharmacologic agents which modify the fate of ligands during receptor-mediated endocytosis has shed considerable light on the pathways involved. Dunn et al.[87] and Tolleshaug and Berg[101] have demonstrated that leupeptin, a tripeptide which effectively inhibits thiol proteases including the cathepsins, specifically inhibits asialofetuin degradation without altering either uptake or transport to the lysosomes. This inhibition occurs in rat liver in vivo, in isolated perfusion, and in isolated rat hepatocytes. Sucrose density gradient fractionation of cells following leupeptin treatment revealed that undegraded ligand moved to and accumulated within lysosomes. Furthermore, leupeptin retarded the continued movement of ligand into the lysosomal compartment, perhaps secondary to an excessive intracellular accumulation of the ligand itself.

Another group of agents, the so-called lysosomotropic amines (e.g., ammonium chloride, chloroquine), are weak bases which freely diffuse and accumulate within lysosomes as well as other acidic compartments as a consequence of being protonated.[102] These agents have no effect on ligand binding or internalization, yet inhibit degradation of ligand, presumably by raising lysosomal pH.[68,103] However, ligand was found to accumulate in a low density prelysosomal compartment, the endosome.[104] Similar findings have been seen with the carboxylic ionophore monensin.[105,106] Tycko and Maxfield[107,108] have demonstrated acidification of a prelysosomal vesicle (endosome) using fluoresceinated asialoorosomucoid. Furthermore, they demonstrated that ligand accumulated within this compartment following exposure to a weak base as a result of neutralization of pH. The role of pH in the intracellular movement of ligand and ASGP-R is discussed in detail below (Section VI).

Independent evidence for endosome-lysosome movement derives from the studies of Hubbard and colleagues. They demonstrated that at ≤20°C delivery of ligand from endosomes to lysosomes is abolished in studies involving subcellular fractionation as well as electron microscopic autoradiography of radiolabeled ligand in perfused rat liver or rat hepatocytes.[58,109] This transfer step is apparently dependent upon the cel-

lular ATP content.[93] This step of ligand transfer or maturation from endosome to lysosome may also be affected by a variety of other compounds including procaine[110] and benzyl alcohol.[111]

Other pharmacologic agents which interfere with intracellular transport, such as colchicine, which promotes disaggregation of microtubules, and cytochalasin B, which inhibits microfilament function, have been examined for effects on the receptor-mediated endocytosis of asialoglycoproteins. Both colchicine and cytochalasin B inhibit ligand degradation, but only slightly increase uptake in isolated hepatocytes.[112] Isopycnic sucrose gradient fractionation revealed that the ligand did not reach the lysosome, but was retained in a low density fraction, presumably the endosome. Similar findings have been reported by Wolkoff et al.[113] Replacement of sodium with potassium in the culture medium prevents ligand delivery to lysosomes, while binding and internalization are unaltered.[114] Although replacement of sodium with sorbital or *N*-methylglucamine at isotonicity decreases ligand uptake by isolated hepatocytes, it is not clear which stage is affected.[23]

Thus, the major pathway for ligand movement during asialoglycoprotein receptor-mediated endocytosis is initiated by ligand binding at the cell surface, internalization within coated pits/coated vesicles, delivery of ligand to endosomal tubules and vesicles in the cell periphery, and transfer/maturation to lysosomes in which degradation occurs (Figure 5). A minor pathway (diacytosis) involves a short circuiting of ligand following internalization such that exocytosis occurs prior to delivery of ligand to lysosomes (Figure 5). A variety of pharmacologic agents have been used to dissect this pathway and to define the role of acidification in the lysosome and endosome and the requirements of temperature for ligand movement.

VI. FATE OF THE RECEPTOR

During receptor-mediated endocytosis, the ligand rapidly leaves the cell surface during internalization en route to the lysosomes. In contrast, the receptor must be spared destruction within the lysosomes. Current evidence supports efficient recycling of the ASGP-R during receptor-mediated endocytosis.

Tolleshaug et al.[68] and Steer and Ashwell,[69] using isolated rat hepatocytes, initially proposed the reutilization of ASGP-R based on observations that in the absence of new protein synthesis, the rate of ligand uptake continued linearly for many hours at 37°C. The quantity of ligand taken up was 72-fold greater than the surface complement of receptors (ligand binding sites) and 4-fold greater than the total cell complement of receptors. Studies by Schwartz et al.[92] with the human hepatoma cell line Hep G2 have reached similar conclusions. These studies supported the observations of Tanabe et al.[18] who proposed that a recycling mechanism existed for the ASGP-R such that the receptor would escape lysosomal destruction. However, their studies suggested that the receptor binding site was localized to the cytoplasmic surface of lysosomal membranes. In light of recent studies of receptor localization and movement (see above, Section IV) it is unlikely that this is the case. Nevertheless, these authors demonstrated that the average half life of the receptor in rat liver was approximately 88 hr, while the ligand was rapidly (within 20 min) catabolized. These observations suggested reutilization of the surface receptor.

Weigel[115] has inferred that the surface receptor is internalized during endocytosis by following the rate of loss of surface binding activity during one cycle of ligand uptake and as a function of temperature. Bridges et al.[75] have provided more direct evidence that the asialoglycoprotein ligand enters the hepatocyte in a complex with its receptor. This was based on the use of a precipitation assay in which intracellular receptor could be resolved into that which was occupied or unoccupied by ligand. In addition, they

suggested that dissociation of ligand and receptor occurred prior to ligand degradation. Harford et al.,[104] have extended these observations in rat hepatocytes in culture by combining the kinetics of ligand movement with the state of ligand (i.e., receptor bound or free) and subcellular fractionation. By use of various inhibitors of receptor-mediated endocytosis (e.g., chloroquine, monensin), they have demonstrated that after leaving the cell surface, ligand and receptor enter a low density prelysosomal endocytic compartment in which receptor-ligand dissociation normally occurs.[105] Baenziger and Fiete[114] have also reported that delivery of ligand to lysosomes is not required for receptor reutilization following treatment of rat hepatocytes with low sodium/high potassium medium. More recently, Fiete et al.[23] have observed a loss of cell surface receptor binding activity following pretreatment of rat hepatocytes with monensin at 37°C. However, in these cells, receptor remained at the cell surface as judged by competition radioimmunoassay and by surface ^{125}I iodination and specific immunoprecipitation. It is difficult to reconcile these series of observations with monensin despite the use of somewhat different drug treatments.

Weigel and Oka[73] have recently assessed the degree of occupancy of the large (50 to 75%) intracellular pool of ASGP-R using ligand binding in rat hepatocytes permeabilized with digitonin.[74] The kinetics of occupancy of intracellular receptors demonstrate that the majority become occupied with ligand in a time- and concentration-dependent manner (70% within 1 to 2 hr at 37°C). In addition, the recovery of cell surface receptor binding activity following a pulse of ligand was more rapid than the recovery of intracellular receptor. This suggests that the intracellular pool of receptor is functional during receptor-mediated endocytosis.

Alternatively, Stockert et al.[76,77] have provided evidence that internal ASGP-R are nonfunctional during receptor-mediated endocytosis. One study was based on perfusion of intact rat liver with antireceptor antibody. This resulted in diminished recovery of surface receptor binding activity.[76] However, the behavior of antibody-receptor complexes may not parallel that of "native" ligand-receptor complexes. This fact obtains in the low density lipoprotein receptor system.[116] In their other study, Stockert et al.[77] demonstrated a loss of binding capacity for asialoorosomucoid following neuraminidase treatment while binding capacity for desialylated ovine submaximallary mucin was maintained at control levels. Since the latter compound exhibits a higher affinity for the receptor than ASOR,[31] new ligand for the ASGP-R could have been generated by the enzyme treatment at the cell surface such that ASOR was inhibited while the high affinity desialylated mucin retained most of its binding to the receptor. At present, therefore, a substantial portion of intracellular ASGP-R most probably function during receptor-mediated endocytosis; however, the size of this pool is not certain in rat hepatocytes.

Schwartz et al.,[92] have established a simple kinetic model for the internalization and recycling of ASGP-R in human hepatoma cells. Using sensitivity of the ASGP-R to external proteases followed by reappearance of ligand binding activity, the fraction of functional receptors on the cell surface (both occupied and unoccupied by ligand) and the fraction within the hepatoma cell (approximately 15 to 20%) were determined. Independent determinations of each of the parameters of ligand-receptor binding, internalization, dissociation of ligand from receptor, and reappearance of unoccupied receptor on the cell surface provided strong support for the direct internalization and recycling of the ASGP-R. This study was recently extended by Ciechanover et al.[117] who demonstrated that internalization of ASOR by hepatoma cells is accompanied by a rapid ($t_{1/2}$ = 0.5 to 1 min) depletion of surface ASGP-R. This is followed by a rapid ($t_{1/2}$ = 2 to 4 min) reappearance of surface receptors, most of which had originated from the original surface receptor pool.

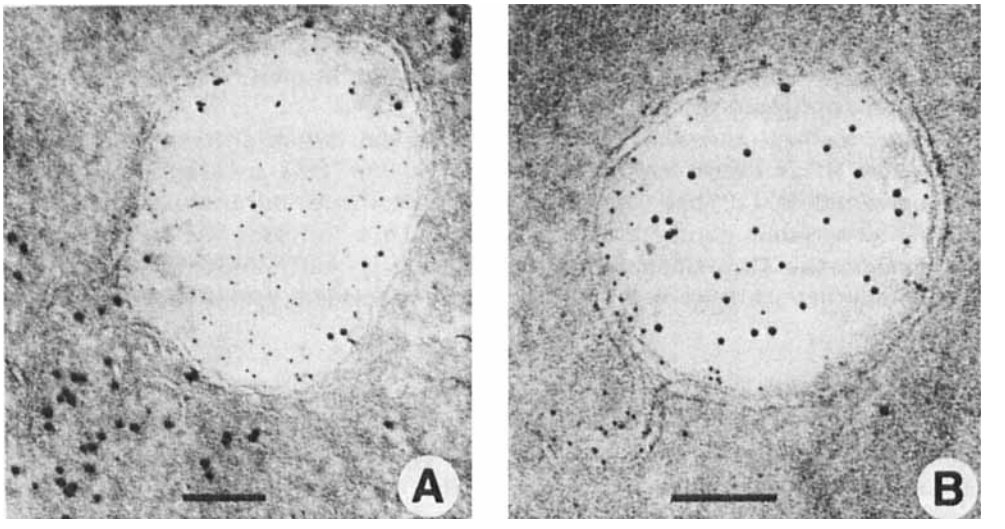

FIGURE 6. Immunocytochemical electron micrograph of the compartment of uncoupling of receptor and ligand (CURL). (A) Immunocytochemical electron micrograph of ultrathin cryosections from perfusion-fixed rat liver during continuous infusion of asialofetuin. Ligand was labeled first with anti-asialofetuin antibody and then with 5 nm colloidal gold-protein A. Thereafter ASGP receptor was immunolabeled with antibody and then with 8 nm colloidal gold-protein A. Free ligand can be seen in the lumen of the vesicular portion of this sorting vesicle, which also shows scarce and heterogeneous receptor distribution. Receptor labeling is intense over the connecting tubules. Bar = 0.1 μm. (B) Similar to (A) except that receptor is labeled with 5 nm gold whereas ligand is labeled with 8 nm gold. Receptor is located predominantly at the fold where a tubule with heavy receptor labeling is connected. Most of the ligand is present free within the vesicle lumen. Bar = 0.1 nm. Adapted from Geuze et al.[64]

Geuze et al.[64] have identified the intracellular site of ASGP-R-ligand uncoupling during receptor-mediated endocytosis (Figure 6). On cryosections of rat liver, the technique of colloidal gold double-label immunoelectron microscopy with antibodies to ASGP-R and ligand allowed these investigators to identify both receptor and ligand initially associated with the membrane of clathrin-coated pits and coated vesicles. Other vesicles were identified which contained ligand within the lumen. The membranes of these vesicles contained few receptors, of note, receptors were concentrated in thin tortuous tubular extensions that were largely free of ligand. This organelle, designated compartment of uncoupling of receptor and ligand (CURL), was the intracellular site at which endocytosed ligand and receptor morphologically segregated prior to the delivery of ligand to lysosomes for degradation (Figure 5). The CURL tubules have recently been defined as the point of entry of ligand-receptor complexes into the sorting vesicle.[90] In addition, the tubules may further serve as intermediates in the return of unoccupied receptors to the cell surface (Figure 5).

The exact mechanisms responsible for ligand-receptor uncoupling are not clear at present. Acidification is one likely candidate since ASGP-R-ligand binding varies tremendously with pH. Hudgin et al.[13] demonstrated a 70% dissociation of bound ligand from the isolated ASGP-R upon lowering the pH from 6.5 to 6.0. Van Lenten and Ashwell[50] made similar observations using ASGP-R within the plasma membrane. Studies of Tycko and Maxfield,[107,108] have demonstrated acidification of an endosomal compartment to an average pH of less than 5.5. This is clearly sufficient to promote ligand-ASGP-R dissociation. However, since the pH measurements represent an average, the uniformity of vesicle acidification is uncertain. Recently, the acidification of this endosomal compartment has been directly demonstrated.[118,119] The compartment of uncoupling ligand and receptor is approximately 14 aℓ (14×10^{-18} ℓ), assuming a

diameter of 0.4 μm[64] and a spherical shape. Independent of buffer, a pH of 6.0 within this compartment theoretically provides approximately 75 H^+ within CURL. Thus, a relatively small number of unbuffered protons injected into this compartment could be sufficient for ligand ASGP-R dissociation.

Another mechanism responsible for uncoupling may include alteration in free Ca^{++} (see Section III). A decrease of 10 μM free Ca^{++} within CURL could be accounted for by a loss of only 80 Ca^{++} ions. This alone is sufficient to provide for substantial ligand-ASGP-R dissociation. Furthermore, a combination of a decrease in Ca^{++} concentration with an increase in H^+ would be more effective than either alteration alone. The relative contribution of each process or that of as yet unrecognized processes remains to be established.

A. Modulation of Receptor Expression

Tolleshaug and Berg[68] found that in the presence of chloroquine or ammonium chloride at 37°C, there was a loss of cell surface receptor binding activity in isolated rat hepatocytes in the absence of added ligand. They concluded that receptors were lost from the cell surface and inhibited from reappearing by these agents, thus suggesting constitutive recycling of the receptor. Schwartz et al.[120] have followed the kinetics of loss and reappearance of surface receptor in the absence or presence of added ligand and lysosomotropic agents. In the absence of ligand, these drugs induced a rapid ($t_{1/2}$ 5.5 to 6 min) loss of surface receptors. In the presence of ligand, the loss of surface receptors was more rapid ($t_{1/2}$ 2.5 to 3 min). The presence of lysosomotropic amines prevented the rapid return of endocytosed receptors to the cell surface. These observations provide indirect evidence for constitutive recycling of ASGP-R. It is not clear whether the pathways involved in constitutive vs. ligand-associated recycling of the ASGP-R are the same or differ in substantial aspects.

In addition to modulation by pharmacologic agents, ASGP-R expression is affected by the growth state of the cells. Gartner et al.[121] demonstrated that uptake (single pass influx) of asialoglycoprotein ligands was reduced in regenerating rat liver, but slowly returned to normal uptake rates following completion of the regeneration process on day 6. Isolated hepatocytes prepared from regenerating rat liver similarly demonstrated reduction of ligand uptake.[122] At the cell surface, ligand binding activity as well as receptor abundance assessed with antireceptor antibody were reduced by 80%, whereas the number of total cell receptors was unchanged. This implied redistribution of a constant number of receptors. Conclusions are limited, however, since the hepatocytes examined may have represented a selected subpopulation of cells due to hepatectomy and regeneration. Additional support for growth-dependent expression of ASGP-R derives from the report of Theilmann et al.[123] which suggests that rapidly dividing human hepatoma cells have less cell surface ligand binding activity than nongrowing cells. Furthermore, Collins et al.,[124] found binding activity in homogenates of livers from fetal mice after day 15 of gestation (term equals 20 days). At birth, ASGP-R activity was consistently detectable and reached adult levels by the fifth post partum day. Assays with antireceptor antibody confirmed the appearance of receptor protein during the rapid growth phase of the neonatal period.

In addition to modulation of the ASGP-R associated with growth, the number of cell surface receptors can be modulated by the presence of ligand. For example, the number of surface receptors can be reduced to 50% the basal level in hepatoma cells after incubation at 37°C with 10 μM asialofetuin.[92] This redistribution of receptors is a direct consequence of the relative rates of the processes involved in receptor recycling. However, the presence of excess ligand does not alter turnover of the receptor protein itself, as demonstrated in hepatoma cells.[25]

The overall pathway of receptor movement during receptor-mediated endocytosis of asialoglycoproteins begins when the receptor, randomly distributed in the sinusoidal plasma membrane, binds ligand and internalizes via a coated pit/vesicle. The receptor ligand complex then enters the endocytic network of tubules/vesicles and is directed to the sorting vesicles (CURL). In this compartment, receptors segregate into the tubules for return to the cell surface (Figure 5). Intracellular pathways involving movement of receptors through the Golgi and the diacytosis of ligand remain speculative (Figure 3).

VII. PERSPECTIVES

The recognition of galactose-terminal glycoproteins via the ASGP-R provides a high efficiency system for targeting macromolecules to the hepatocyte. Fiume and associates[125-127] initially demonstrated that conjugates of antiviral agents (e.g., trifluorothymidine or Ara A) coupled to either asialofetuin or lactosylated albumin resulted in a complex which was capable of dramatically reducing Ectromelia viral DNA synthesis in mice in vivo. Attie et al.[128] similarly made use of the ASGP-R system to selectively deliver an essential cell nutrient, cholesterol, to hepatocytes via low density lipoprotein-lactose conjugates. In addition, hybrid molecules have been constructed using diphtheria toxin fragment A conjugated to asialofetuin[129] or asialoorosomucoid.[130] These hybrid molecules demonstrated specificity for the ASGP-R, with cellular uptake and cytotoxicity limited to the hepatocyte.[129,130] In the future, this approach may extend to site-specific chemotherapy. More recently, Wu et al.,[131] have used the ASGP-R system to selectively rescue hepatocytes from the generalized toxicity of a chemotherapeutic agent (methotrexate). Folinic acid, which bypasses the methotrexate inhibition of dihydrofolate reductase, was conjugated to asialofetuin and administered to cells grown in the presence of methotrexate. Hepatoma cells which express the ASGP-R were selectively rescued from methotrexate-induced cell death, whereas cells lacking the ASGP-R were not rescued.[131]

Many questions remain unanswered in regard to the structure and function of the hepatic ASGP-R. Structural characterization of the receptor subunits will require complete protein sequence data. These and additional data will be necessary to define the receptor topology with respect to the plasma membrane. What and where are the post-translational modifications and do they influence receptor function? What is the precise molecular organization of the receptor within the plasma membrane — how are the subunits organized? Are additional modifying units responsible for ligand binding? What is the stoichiometry of ligand binding for the functional receptor unit? How does Ca^{++} regulate ligand binding? Another broad series of unanswered questions relate to the function of the ASGP-R and the basis for receptor-mediated endocytosis. What structural elements are responsible for receptor concentration within coated pits? How does ligand binding to receptor influence internalization? What are the dynamics and pathways of constitutive receptor recycling? What mechanisms are responsible for dissociation and segregation of ligand and receptor during receptor-mediated endocytosis? What governs these various intracellular sorting mechanisms? How do these questions regarding the ASGP-R relate to other systems of receptor-mediated endocytosis?

During the past 15 years a substantial body of knowledge has accumulated regarding the biochemistry of the hepatic asialoglycoprotein receptor. The general mechanisms which govern its recognition of galactose-terminal ligands and the subsequent receptor-mediated endocytosis establish this receptor as a valuable model system. Certainly future studies with the hepatic asialoglycoprotein receptor will continue to critically address these and other important issues.

ACKNOWLEDGMENTS

The author is indebted to A. J. Ciechanover and C. F. Simmons for their constructive comments and H. J. Geuze for the electron micrographs. A. L. S. was supported during the course of this writing by N. I. H. (GM 32477), the National Foundation (Basil O'Connor Grant), and as a John A. and George Hartford Foundation Fellow.

REFERENCES

1. Ashwell, G. and Morell, A., The role of surface carbohydrates in the hepatic recognition and transport of circulating glycoproteins, *Adv. Enzymol.* 41, 99, 1974.
2. Morell, A., Irvine, R. A., Sternlieb, I., Scheinberg, I. H., and Ashwell, G., Physical and chemical studies on ceruloplasmin. V. Metabolic studies on sialic acid free ceruloplasmin *in vivo*, *J. Biol. Chem.*, 243, 155, 1968.
3. Hickman, J., Ashwell, G., Morell, A. G., Van der Hamer, C. J. A., and Scheinberg, I. H., Physical and chemical studies on ceruloplasmin. VIII. Preparation of N-acetylneuraminic acid-1-^{14}C labeled ceruloplasmin, *J. Biol. Chem.*, 245, 759, 1970.
4. Gregoriadis, G., Morell, A. G., Sternlieb, I., and Scheinberg, I. H., Catabolism of desialylated ceruloplasmin in the liver, *J. Biol. Chem.*, 245, 5833, 1970.
5. Ashwell, G. and Harford, J., Carbohydrate receptors of the liver, *Annu. Rev. Biochem.*, 51, 531, 1982.
6. Stockert, R. J., Morell, A. G., and Scheinberg, I. H., Mammalian hepatic lectin, *Science*, 186, 365, 1974.
7. Neufeld, E. and Ashwell, G., Carbohydrate recognition systems for receptor-mediated pinocytosis, in *The Biochemistry of Glycoproteins and Proteoglycans*, Lennarz, W., Ed., Plenum Press, New York, 1980, 241.
8. Stahl, P., Mannose-specific carbohydrate receptors in *Receptors and Recognition*, Cautrecasas, P., Ed., Pitman, 1983.
9. Regoeczi, E. and Hatton, M. W. C., Asialoglycoprotein clearance in the chicken: evidence for the lack of a hepatic asialoglycoprotein receptor, *Can. J. Physiol. Pharmacol.*, 54, 27, 1976.
10. Lunney, J. and Ashwell, G., A hepatic receptor of avian origin capable of binding specifically modified glycoproteins, *Proc. Natl. Acad. Sci. U.S.A.*, 73, 341, 1976.
11. Ashwell, G. and Steer, C. J., Hepatic recognition and catabolism of serum glycoproteins, *JAMA*, 246, 2358, 1981.
12. Morell, A. G. and Scheinberg, I. H., Solubilization of hepatic binding sites for asialoglycoproteins *Biochem. Biophys. Res. Commun.*, 48, 808, 1972.
13. Hudgin, R. L., Pricer, W. E., Jr., Ashwell, G., Stockert, R. J., and Morell, A. G., The isolation and properties of a rabbit liver binding protein specific for asialoglycoproteins, *J. Biol. Chem.*, 249, 5536, 1974.
14. Connolly, D. T., Hoppe, C. A., Hobish, M. K., and Lee, Y. C., Steady state and kinetic analysis of the binding of asialoorosomucoid to the isolated rabbit hepatic lectin, *J. Biol. Chem.*, 256, 12940, 1981.
15. Andersen, T. T., Freytag, J. W., and Hill, R. L., Physical studies of the rabbit hepatic galactoside-binding protein, *J. Biol. Chem.*, 257, 8036, 1982.
16. Kawasaki, T. and Ashwell, G., Chemical and physical properties of an hepatic membrane protein that specifically binds asialoglycoproteins, *J. Biol. Chem.*, 251, 1296, 1976.
17. Steer, C. J., Kempner, E. S., and Ashwell, G., Molecular size of the hepatic receptor for asialoglycoproteins determined in situ by radiation inactivation, *J. Biol. Chem.*, 256, 5851, 1981.
18. Tanabe, T., Pricer, W. E., Jr., and Ashwell, G., Subcellular membrane topology and turnover of a rat hepatic binding protein specific for asialoglycoproteins, *J. Biol. Chem.*, 254, 1038, 1979.
19. Sawamura, T., Nakada, H., Fujii-Kuriyama, Y., and Tashiro, Y., Some properties of a binding protein specific for asialoglycoproteins and its distribution in rat liver microsomes, *Cell Struct. Funct.*, 5, 133, 1980.
20. Warren, R. and Doyle, D., Turnover of the surface proteins and the receptor for serum asialoglycoproteins in primary cultures of rat hepatocytes, *J. Biol. Chem.*, 256, 1346, 1981.

21. Schwartz, A. L., Marshak-Rothstein, A., Rup, D., and Lodish, H. F., Identification and quantification of the rat hepatocyte asialoglycoprotein receptor, *Proc. Natl. Acad. Sci. U.S.A.*, 78, 3348, 1981.
22. Harford, J., Lowe, M., Tsunoo, H., and Ashwell, G., Immunological approaches to the study of membrane receptors, *J. Biol. Chem.*, 257, 12685, 1982.
23. Fiete, K., Brownell, M. D., and Baenziger, J. U., Evidence for transmembrane modulation of the ligand-binding site of the hepatocyte galactose/N-acetylgalactosamine-specific receptor, *J. Biol. Chem.*, 258, 817, 1983.
24. Baenziger, J. U. and Maynard, Y., Human hepatic lectin, *J. Biol. Chem.*, 255, 4607, 1980.
25. Schwartz, A. L. and Rup, D., Biosynthesis of the human asialoglycoprotein receptor, *J. Biol. Chem.*, 258, 11249, 1983.
26. Pricer, W. E. and Ashwell, G., Subcellular distribution of a mammalian hepatic binding protein specific for asialoglycoproteins, *J. Biol. Chem.*, 251, 7539, 1976.
27. Klausner, R. D., Bridges, K., Tsunoo, H., Bumenthal, R., Weinstein, J. N., and Ashwell, G., Reconstitution of the hepatic asialoglycoprotein receptor with phospholipid vesicles, *Proc. Natl. Acad. Sci. U.S.A.*, 77, 5087, 1980.
28. Kawasaki, T. and Ashwell, G., Carbohydrate structure of glycopeptides isolated from a hepatic membrane-binding protein specific for asialoglycoproteins, *J. Biol. Chem.*, 251, 5292, 1976.
29. Paulson, J. C., Hill, R. L., Tanabe, T., and Ashwell, G., Reactivation of asialo-rabbit liver binding protein by resialylation with β-D-galactoside α2-6 sialyltransferase, *J. Biol. Chem.*, 252, 8624, 1977.
30. Pricer, W. E. and Ashwell, G., The binding of desialylated glycoproteins by plasma membranes of liver, *J. Biol. Chem.*, 246, 4825, 1971.
31. Stockert, R., Morell, A. G., and Scheinberg, I. H., Hepatic binding protein and the protective role of its sialic acid residues, *Science*, 186, 667, 1977.
32. Harford, J. and Ashwell, G. G., Immunological evidence for the transmembrane nature of the rat liver receptor for asialoglycoproteins, *Proc. Natl. Acad. Sci. U.S.A.*, 78, 1557, 1981.
33. Kawasaki, T. and Ashwell, G., Isolation and characterization of an avian hepatic binding protein specific for N-acetylglucosamine-terminated glycoproteins, *J. Biol. Chem.*, 255, 6536, 1977.
34. Drickamer, K., Complete amino acid sequence of an avian receptor for glycoproteins, *J. Biol. Chem.*, 256, 5827, 1981.
35. Drickamer, K. and Mamon, J. F., Phosphorylation of a membrane receptor for glycoproteins, *J. Biol. Chem.*, 257, 15156, 1982.
36. Nakada, H., Sawamura, T., and Tashior, Y., Biosynthesis and insertion of a hepatic binding protein specific for asialoglycoproteins into endoplasmic reticulum membranes, *J. Biochem.*, 89, 135, 1981.
37. Sarkar, M., Liao, J., Kabat, E. A., Tanabe, T., and Ashwell, G., The binding site of rabbit hepatic lectin, *J. Biol. Chem.*, 25, 3170, 1979.
38. Stowell, C. P. and Lee, Y. C., Neoglycoproteins: the preparation and application of synthetic glycoproteins, *Adv. Carbohydr. Chem. Biochem.*, 37, 225, 1980.
39. Stowell, C. P. and Lee, Y. C., The binding of d-glucosyl-neoglycoproteins to the hepatic asialoglycoprotein receptor, *J. Biol. Chem.*, 253, 6107, 1978.
40. Stowell, C. P., Lee, R. T., and Lee, Y. C., Studies on the specificity of rabbit hepatic carbohydrate-binding protein using neoglycoproteins, *Biochemistry*, 19, 4904, 1980.
41. Lee, R. T., Binding site of the rabbit liver lectin specific for galactose/N-acetylgalactosamine, *Biochemistry*, 21, 1045, 1982.
42. Lee, R. T., Meyers, R. T., and Lee, Y. C., Further studies on the binding characteristics of rabbit liver galactose/*N*-acetylgalactosamine-specific lectin, *Biochemistry*, 21, 6292, 1982.
43. Fornet, B., Montreiul, J., Strecker, G., Durland, L., Haverkamp, J., Vliegenthart, J., Binett, J., and Schmid, K., Determination of the primary structures of 16 asialo-carbohydrate units derived from human plasma, α_1-acid glycoprotein by 360-MH2'H NMR spectroscopy and permethylation analysis, *Biochemistry*, 17, 5206, 1978.
44. Krantz, M. J., Holtman, N., Stowell, C., and Lee, Y. C., Attachment of thioglycosides to proteins: enhancement of liver membrane binding, *Biochemistry*, 15, 3963, 1976.
45. Lee, R. and Lee, Y., Preparation and some biochemical properties of neoglycoproteins produced by reductive amination of thioglycosides containing an N-aldehydroaglycon, *Biochemistry*, 19, 156, 1980.
46. Baenziger, J. U. and Fiete, D., Galactose and N-acetylgalactosamine-specific endocytosis of glycopeptides by isolated rat hepatocytes, *Cell*, 22, 611, 1980.
47. Connolly, D. T., Townsend, R. R., Kawaguchi, K., Bell, W. R., and Lee, Y. C.., Binding and endocytosis of cluster glycosides; evidence for a short-circuit pathway that does not lead to degradation, *J. Biol. Chem.*, 257, 939, 1982.
48. Kawaguchi, K., Kuhlenschmidt, M., Roseman, S., and Lee, Y. C., Different uptake of D-galactosyl- and D-glycosyl-neoglycoproteins by isolated rat hepatocytes, *J. Biol. Chem.*, 256, 2230, 1981.

49. Lee, Y. C., Townsend, R. R., Hardy, M. R., Lonngren, J., Arnarp, J., Haraldsson, J., and Lonn, H., Binding of synthetic oligosaccharides to the hepatic Gal/GalNAc lectin, *J. Biol. Chem.*, 258, 199, 1983.
50. Van Lenten, L. and Ashwell, G., The binding of desialylated glycoproteins by plasma membrane of rat liver, *J. Biol. Chem.*, 247, 4633, 1972.
51. Tolleshaug, H. and Berg, T., Investigation of the uptake of asialoglycoproteins by isolated rat hepatocytes implications for its biological significance, *Hoppe-Seyler's Z. Physiol. Chem.*, 361, 1155, 1980.
52. Weigel, P. H., Characterization of asialoglycoprotein receptor on isolated rat hepatocytes, *J. Biol. Chem.*, 255, 6111, 1980.
53. Blomhoff, R., Tolleshaug, H., and Berg, T., Binding of calcium ions to the isolated asialoglycoprotein receptor, *J. Biol. Chem.*, 257, 7456, 1982.
54. Blumental, R., Klausner, R. D., and Weinstein, J. N., Voltage-dependent translocation of the asialoglycoprotein receptor across lipid membranes, *Nature*, 288, 333, 1980.
55. Hubbard, A. L., Wilson, G., Ashwell, G., and Stukenbrok, H., An electron microscope autoradiographic study of the carbohydrate recognition systems in rat liver. I. Distribution of ^{125}I-ligand among the liver cell types, *J. Cell. Biol.*, 83, 47, 1979.
56. Hubbard, A. L., and Stukenbrok, H., An electron microscope autoradiographic study of the carbohydrate recognition systems in rat liver. II. Intracellular fates of the ^{125}I-ligands, *J. Cell. Biol.*, 83, 65, 1979.
57. Tolleshaug, H., Berg, T., Nilsson, M., and Norum, K. R., Uptake and degradation of ^{125}I-labeled asialo-fetuin by isolated rat hepatocytes, *Biochim. Biophys. Acta*, 499, 73, 1977.
57a. Partridge, W. M., Van Herle, A. J., Naruse, R. T., Fierer, G., and Costin, A., *In Vivo* quantification of receptor-mediated uptake of asialoglycoproteins by rat liver, *J. Biol. Chem.*, 258, 990, 1983.
58. Wall, D. A. and Hubbard, A. L., Galactose-specific recognition system of mammalian liver: receptor distribution on the hepatocyte cell surface, *J. Cell Biol.*, 90, 687, 1981.
59. Stockert, R. J., Haimes, H. B., Morell, A. G., Novikoff, P. M., Novikoff, A. B., Quintana, N., and Sternlieb, I., Endocytosis of asialoglycoprotein-enzyme conjugates by hepatocytes, *Lab. Invest.*, 43, 556, 1980.
60. Hardonk, M. J. and Scholtens, H. B., A histochemical study about the zonal distribution of the galactose-binding protein in rat liver, *Histochemistry*, 69, 289, 1980.
61. Scholtens, H. B., Meijer, D. K. F., and Hardonk, M. J., A histochemical study on the distribution of injected canine intestinal alkaline phosphatase in rat liver, *Liver*, 2, 14, 1982.
62. Matsuura, S., Nakada, H., Sawamura, T., and Tashiro, Y., Distribution of an asialoglycoprotein receptor on rat hepatocyte cell surface, *J. Cell. Biol.*, 95, 864, 1982.
63. Geuze, H. J., Slot, J. W., Strous, G. J. A. M., Lodish, H. F., and Schwartz, A. L., Immunocytochemical localization of the receptor for asialoglycoprotein in rat liver cells, *J. Cell. Biol.*, 92, 865, 1982.
64. Geuze, H. J., Slot, J. W., Strous, G. J. A. M., Lodish, H. F., and Schwartz, A. L., Intracellular site of asialoglycoprotein receptor-ligand uncoupling: double-label immunoelectron microscopy during receptor mediated endocytosis, *Cell*, 32, 277, 1983.
65. Zeitlin, P. L. and Hubbard, A. L., Cell surface distribution and intracellular fate of asialoglycoproteins: a morphological and biochemical study of isolated rat hepatocytes and monolayer cultures, *J. Cell. Biol.*, 92, 634, 1982.
66. Weigel, P. H., Rat hepatocytes bind to synthetic galactoside surfaces via a patch of asialoglycoprotein receptors, *J. Cell. Biol.*, 87, 855, 1980.
67. Carlsen, S. A., Schmell, E., Weigel, P. H., and Roseman, S., The effect of the method of isolation on the surface properties of isolated rat hepatocytes, *J. Biol. Chem.*, 256, 8058, 1981.
68. Tolleshaug, H. and Berg, T., Chloroquine reduces the number of asialoglycoprotein receptors in the hepatocyte plasma membrane, *Biochem. Pharmacol.*, 28, 2919, 1979.
69. Steer, C. J. and Ashwell, G., Studies on a mammalian hepatic binding protein specific for asialoglycoproteins, evidence for receptor recycling in isolated rat hepatocytes, *J. Biol. Chem.*, 255, 3008, 1980.
70. Schwartz, A. L., Rup, D., and Lodish, H. F., Difficulties in the quantification of asialoglycoprotein receptors on the rat hepatocyte, *J. Biol. Chem.*, 255, 9033, 1980.
71. Weigel, P. H. and Oka, J. A., Endocytosis and degradation mediated by the asialoglycoprotein receptor in isolated rat hepatocytes, *J. Biol. Chem.* 257, 1201, 1982.
72. Weigel, P. H. and Oka, J. A., The surface content of asialoglycoprotein receptors on isolated hepatocytes is reversibly modulated by changes in temperature, *J. Biol. Chem.*, 258, 5089, 1983.
73. Weigel, P. H. and Oka, J. A., The large intracellular pool of asialoglycoprotein receptors functions during the endocytosis of asialoglycoproteins by isolated rat hepatocytes, *J. Biol. Chem.*, 258, 5095, 1983.

74. Weigel, P. H., Ray, D. A., and Oka, J. A., Quantitation of intracellular membrane-bound enzymes and receptors in digitonin-permeabilized cells, *Anal. Biochem.*, 133, 437, 1983.
75. Bridges, K., Harford, J., Ashwell, G., and Klausner, R. D., Fate of receptor and ligand during endocytosis of asialoglycoproteins by isolated hepatocytes, *Proc. Natl. Acad. Sci. U.S.A.*, 79, 350, 1982.
76. Stockert, R. J., Gartner, U., Morell, A. G., and Wolkoff, A. W., Effects of receptor-specific antibody on the uptake of desialylated glycoproteins in the isolated perfused rat liver, *J. Biol. Chem.*, 255, 3830, 1980.
77. Stockert, R. J., Howard, K. J., Morell, A. G., and Scheinberg, H. I., Functional segregation of hepatic receptors for asialoglycoproteins during endocytosis, *J. Biol. Chem.*, 255, 9028, 1980.
78. Slot, J. W. and Geuze, H. J., Immunoelectron microscopic exploration of the golgi complex, *J. Histochem. Cytochem.*, 31, 1049, 1983.
79. Kolb, H., Schudt, C., Kolb-Bachofen, V., and Kolb, H. A., Cellular recognition by rat liver cells of neuraminidase-treated erythrocytes, *Exp. Cell Res.*, 113, 319, 1978.
80. Schlepper-Schafer, J., Kolb-Bachofen, V., and Kolb, H., Analysis of lectin-dependent recognition of asialo-erythrocytes by Kupffer cells, *Biochem. J.*, 186, 827, 1980.
81. Muller, E., Franco, M. W., and Schauer, R., Involvement of membrane galactose in the *in vivo* and *in vitro* sequestration of desialylated erythrocytes, *Hoppe-Seyler's Z. Physiol. Chem.*, 362, 1615, 1981.
82. Kolb-Bachofen, V., Schepper-Schafer, J., and Vogell, W., Eletron microscopic evidence for an asialoglycoprotein receptor on Kupffer cells: localization of lectin-mediated endocytosis, *Cell*, 29,859, 1982.
83. Kolb-Bachofen, V., Schlepper-Schafer, J., Teradera, R., Vogt,D., and Kolb, H., D-Galactose-specific lectin on rat Kupffer cells — its role in the biology and pathology of the liver, in *Structural Carbohydrates in the Liver*, Popper, H., Ed., MTP Press, Great Britain, 1983, 277.
84. Schwartz, A. L. Fridovich, S. E., Knowles, B. B., and Lodish, H. F., Characterization of the asialoglycoprotein receptor in a continuous hepatoma line, *J. Biol. Chem.*, 256, 8878, 1981.
85. Wall, D. A., Wilson, G., and Hubbard, A. L., The galactose-specific recognition system of mammalian liver: the route of ligand internalization in rat hepatocytes, *Cell*, 21, 79, 1980.
86. LaBadie, J. H., Peterson-Chapman, K., and Aronson, N. N., Jr., Glycoprotein catabolism in rat liver, *Biochem. J.*, 152, 271, 1975.
87. Dunn, W. A., LaBadie, J. H., and Aronson, N. N., Inhibition of ^{125}I-asialofetuin catabolism by leupeptin in the perfused rat liver and *in vivo*, *J. Biol. Chem.*, 254, 4191, 1979.
88. Tolleshaug, H., Berg, T., Frolich, W., and Norum, K. R., Intracellular localization and degradation of asialofetuin in isolated rat hepatocytes, *Biochim. Biophys. Acta*, 585, 71, 1979.
89. Quintart, J., Courtoy, P.-J., Limet, J.- N., and Baudhuin, P., Galactose-specific endocytosis in rat liver, *Eur. J. Biochem.*, 131, 105, 1983.
90. Geuze, H. J., Slot, J. W., Strous, G. J. A. M., and Schwartz, A. L., The pathway of the asialoglycoprotein-ligand during receptor-mediated endocytosis: a morphological study with colloidal gold/ligand in the human hepatoma cell line, Hep G2, *Eur. J. Cell. Biol.*, 32, 38, 1983.
91. Tolleshaug, H., Berg, T., and Holte, K., Kinetics of internalization and degradation of asialo-glycoproteins in isolated rat hepatocytes, *Eur. J. Cell. Biol.*, 23, 104, 1980.
92. Schwartz, A. L., Fridovich, S. E., and Lodish, H. F., Kinetics of internalization and recycling of the asialoglycoprotein receptor in a hepatoma cell line, *J. Biol. Chem.*, 257, 4230, 1982.
93. Tolleshaug, H., Kolset, S. O., and Berg, T., The influence of cellular energy levels on receptor-mediated endocytosis and degradation of asialoglycoproteins in suspended hepatocytes, unpublished observations.
94. Young, S. P., Bomfod, A., and Williams, R., Dual pathway for the uptake of rat asialotransferrin by rat asialotransferrin by rat hepatocytes, *J. Biol. Chem.*, 258, 4972, 1983.
95. Regoeczi, E., Chindemi, P. A., Debanne, M. T., and Hatton, W. C., Dual nature of the hepatic lectin pathway for human asialotransferrin type 3 in the rat, *J. Biol. Chem.*, 257, 5431, 1982.
96. Tolleshaug, H., Chindemi, P. A., and Regoeczi, E., Diacytosis of human asialotransferrin type 3 by isolated rat hepatocytes, *J. Biol. Chem.*, 256, 6526, 1981.
97. Klausner, R. D., Van-Reuswoude, J., Ashwell, G., Dean, A., Schechter, A., and Bridges, K. R., Receptor-mediated endocytosis of transferrin in K562 cells, *J. Biol. Chem.*, 258, 4715, 1983.
98. Ciechanover, A. J., Schwartz, A. L., Dautry, A., and Lodish, H. F., Kinetics of internalization and recycling of transferrin and the transferrin receptor in a human hepatoma cell line, *J. Biol. Chem.*, 258, 9861, 1983.
99. Regoeczi, E., Chindemi, P. A., Debanne, M. T., and Charlwood, P. A., Partial resialylation of human asialotransferrin type 3 in the rat, *Proc. Natl. Acad. Sci. U.S.A.*, 79, 2260, 1982.
99a. Simmons, C. F. and Schwartz, A. L., Recycling of ligand during receptor-mediated transport of asialoglycoproteins, *J. Cell Biol.*, 97, 428a, 1983.

100. Townsend, R. R., Wall, D. A., Hubbard, A. L., and Lee, Y. C., Rapid release of galactose-terminated ligands after endocytosis by hepatic parenchymal cells: evidence for a role of carbohydrate structure in the release of internalized ligand from receptor, *Proc. Natl. Acad. Sci. U.S.A.*, 81, 466, 1984.
101. Tolleshaug, H. and Berg, T., The effect of leupeptin on intracellular digestion of asialofetuin in rat hepatocytes, *Exp. Cell. Res.*, 134, 207, 1981.
102. Ohkuma, S. and Poole, B., Fluorescence probe measurements of the intralysosome pH in living cells and the perturbation of pH by various agents, *Proc. Natl. Acad. Sci. U.S.A.*, 75, 3327, 1978.
103. Berg, T. and Tolleshaug, H., The effects of ammonium ions and chloroquine on uptake and degradation of ^{125}I-labeled asialo-fetuin in isolated rat hepatocytes, *Biochem. Pharmacol.*, 29, 917, 1980.
104. Harford, J., Bridges, K., Ashwell, G., and Klausner, R. D., Intracellular dissociation of receptor-bound asialoglycoproteins in cultured hepatocytes, *J. Biol. Chem.*, 258, 3191, 1983.
105. Harford, J., Wolkoff, A. W., Ashwell, G., and Klausner, R. D., Monensin inhibits intracellular dissociation of asialoglycoproteins from their receptor, *J. Cell. Biol.*, 96, 1824, 1983.
106. Berg, T., Blomhoff, R., Naess, L., Tolleshaug, H., and Drevon, C. A., Monensin inhibits receptor-mediated endocytosis in rat hepatocytes, *Exp. Cell Res.*, 148, 319, 1983.
107. Tycko, B. and Maxfield, F. R., Rapid acidification of endoytic vesicles containing a_2-macroglobulin, *Cell*, 28, 643, 1982.
108. Tycko, B., Keith, C. H., and Maxfield, F. R., Rapid acidification of endocytic vesicles containing asialoglycoprotein in cells of a human hepatoma line, *J. Cell. Biol.*, 97, 1762, 1983.
109. Dunn, W. A., Hubbard, A. L., and Aronson, N. N., Low temperature selectively inhibits fusion between pinocytotic vesicles and lysosomes during heterophagy of ^{125}I-asialofetuin by the perfused rat liver, *J. Biol. Chem.*, 255, 5971, 1980.
110. Tolleshaug, H., Berg, T., and Holte, K., Effects of local anesthetics and related compounds on the endocytosis and catabolism of asialoglycoproteins in isolated hepatocytes, *Biochim. Biophys. Acta*, 714, 114, 1982.
111. Tolleshaug, H. and Berg, T., Evidence for the selective inhibition of fusion between endocytic vesicles and lysosomes by benzyl alcohol, *Biochem. Pharmacol.*, 31, 593, 1982.
112. Kolset, S. O., Tolleshaug, H., and Berg, T., The effects of colchicine and cytochalasin B on uptake and degradation of asialo-glycoproteins in isolated rat hepatocytes, *Exp. Cell. Res.*, 122, 159, 1979.
113. Wolkoff, A. W., Harford, J., Ashwell, G., and Klausner, R. D., Intracellular segregation of asialoglycoproteins and their receptor: a prelysosomal event subsequent to dissociation of the ligand-receptor complex, *J. Cell. Biol.*, 98, 375, 1984.
114. Baenziger, J. U. and Fiete, D., Recycling of the hepatocyte asialoglycoprotein receptor does not require delivery of ligand to lysosomes, *J. Biol. Chem.*, 257, 6007, 1982.
115. Weigel, P., Evidence that the hepatic asialoglycoprotein receptor is internalized during endocytosis and that receptor recycling can be uncoupled from endocytosis at low temperature, *Biochem. Biophys. Res. Commun.*, 101, 1419, 1981.
116. Anderson, R. G. W., Brown, M. S., Beisiegel, U., and Goldstein, J. L., Surface distribution and recycling of the low density lipoprotein receptor as visualized with anti-receptor antibodies, *J. Cell. Biol.*, 96, 523, 1982.
117. Ciechanover, A., Schwartz, A. L., and Lodish, H. F., The asialoglycoprotein receptor internalizes and recycles independently of the transferrin and insulin receptors, *Cell*, 32, 267, 1983.
118. Galloway, C. J., Dean, G. E., March, M., Rudnick, G., and Mellman, I., Acidification of macrophage and fibroblast endocytic vesicles, *in vitro*, *Proc. Natl. Acad. Sci. U.S.A.*, 80, 3334, 1983.
119. Stone, D. K., Xie, X.-S., and Racker, E., An ATP-driven proton pump in clathrin-coated vesicles, *J. Biol. Chem.*, 258, 4059, 1983.
120. Schwartz, A. L., Bolognesi, A., and Fridovich, S. E., Recycling of the asialoglycoprotein receptor and the effect of lysosomotropic amines in hepatoma cells, *J. Cell. Biol.*, 98, 732, 1984.
121. Gartner, U., Stockert, R., Morell, A. G., and Wolkoff, A. W., Modulation of the transport of bilirubin and asialoorosomucoid during liver regeneration, *Hepatology*, 1, 99, 1981.
122. Howard, D. J., Stockert, R. J., and Morell, A. G., Asialoglycoprotein receptors in hepatic regeneration, *J. Biol. Chem.*, 257, 2856, 1982.
123. Theilmann, L., Teicher, L., Schildkraut, C. S., and Stockert, R. J., Growth-dependent expression of a cell surface glycoprotein, *Biochim. Biophys. Acta*, 762, 475, 1983.
124. Collins, J. C., Stockert, R. J., and Morell, A. G., Asialoglycoprotein receptor expression in murine pregnancy and development, *Hepatology*, 4, 80, 1984.
125. Fiume, L., Mattioli, A., Balboni, P. G., Tognon, M., Barbanti-Brodano, G., De Vries, J., and Wieland, Th., Enhanced inhibition of virus DNA synthesis in hepatocytes by trifluorothymidine coupled to asialofetuin, *FEBS Lett.*, 103, 47, 1979.

126. Fiume, L., Mattioli, A., Busi, C., Balboni, P. G., Barbanti-Broadano, G., De Vries, J., Altmann, R., and Wieland, Th., Selective inhibition of ectromelia virus DNA synthesis in hepatocytes by adenine-9-β-D-arabinofuranoside (Ara-A) and adenine-9-β-D-arabinofuranoside 5'-monophosphate (Ara-AMP) conjugated to asialofetuin, *FEBS Lett.*, 116, 185, 1980.
127. Fiume, L., Busi, C., Mattioli, A., Balboni, P. G., and Barbanti-Brodano, G., Hepatocyte targeting of adenine-9-β-D-arabinofuranoside 5'-monophosphate (ara-AMP) coupled to lactosaminated albumin, *FEBS Lett.*, 129, 261, 1981.
128. Attie, A. D., Pittman, R. C., and Steinberg, D., Metabolism of native and of lactosylated human low density lipoprotein: evidence for two pathways for catabolism of exogenous proteins in rat hepatocytes, *Proc. Natl. Acad. Sci. U.S.A.*, 77, 5923, 1980.
129. Cawley, D. B., Simpson, D. L., and Herschman, H. R., Asialoglycoprotein receptor mediates the toxic effects of an asialofetuin-diphtheria toxin fragment A conjugate on cultured rat hepatocytes, *Proc. Natl. Acad. Sci. U.S.A.*, 78, 3383, 1981.
130. Chang. T.-M. and Kullberg, D. W., Studies of the mechanism of cell intoxication by diphtheria toxin fragment A-asialoorosomucoid hybrid toxins, *J. Biol. Chem.*, 257, 12563, 1982.
131. Wu, G.Y., Wu, C. H., and Stockert, R. J., A model for specific rescue of normal hepatocytes during methotrexate treatment of hepatic malignancy, *Proc. Natl. Acad. Sci. U.S.A.*, 80, 3078, 1983.