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Cellular Pathways of Galactose-Terminal Ligand Movement in a Cloned Human Hepatoma Cell Line

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SUMMARY

The intracellular pathways taken by galactose-terminal glycoproteins were examined following endocytosis by the asialoglycoprotein receptor in monolayers of the human hepatoma cell line, Hep G2. In addition to a pathway leading to lysosomal degradation, single cohort kinetics revealed that up to 28% of surface-bound and internalized ^{125}I-asialoorosomucoid (ASOR) eventually returned undegraded to the extracellular medium over 6 hr in the presence or absence of free ASOR in the exocytosis medium. This reappearance of ligand in the exocytosis medium represented a constant fraction of surface bound and internalized ^{125}I-ASOR, and followed pseudo-first order kinetics with $t_{1/2}$ = 84 min (long transit pool). Under conditions of enhanced ligand-receptor dissociation (incubation with 100 mM *N*-acetylgalactosamine (GalNAc)), at least 50% of initially internalized ^{125}I-ASOR returned to the cell surface as ligand-receptor complexes, followed by dissociation of free ligand into the exocytosis medium. This rapid transit pool of ligand also displayed pseudo-first order kinetics with $t_{1/2}$ = 24 min. Exocytosis of ^{125}I-Gal-cytochrome *c*, a synthesized ligand displaying rapid dissociation from the asialoglycoprotein receptor (ASGP-R), paralleled the kinetics of the rapid transit pool of ^{125}I-ASOR ($t_{1/2}$ = 28 min). Furthermore, in addition to spontaneous dissociation from ASPG-R following return to the cell surface, studies conducted in saponin-permeabilized monolayers support the return of free intracellular ^{125}I-Gal-cytochrome *c* to the cell surface during exocytosis. The rapid transit pool of ligand was insensitive to inhibition by 10 mM sodium azide or 0.1 mM primaquine. In contrast, the long transit pool destined for exocytosis was inhibited 50% by 10 mM sodium azide, but insensitive to inhibition by 0.1 mM primaquine. These data suggest that, following internalization by the ASGP-R, a major pathway of ligand movement includes the rapid return of ligand-receptor complexes and/or free ligand to the cell surface. Return of ligand-receptor complexes or free ligand to the cell surface occurs prior to an acidic sorting compartment, can involve multiple cycles of return to the cell surface, and may involve passage through other nonlysosomal intracellular organelles.

INTRODUCTION

Many hormones, growth factors, and other macromolecular ligands gain entry to intracellular compartments by a process known as receptor-mediated endocytosis. Binding of ligand molecules to specific receptors on the cell surface leads to internalization of receptor-ligand complexes from specialized regions of plasma membrane called coated pits, followed by formation of coated vesicles and endosomes. Sorting of the contents and membrane-bound components of endosomes allows selective targeting of receptors and ligands to their ultimate intracellular destinations. Some ligands appear ultimately to enter the cytoplasm, such as the core of Semliki Forest virus after entry into fibroblasts (1); other ligands, such as low density lipoproteins, eventually enter the lysosomal compartment and are degraded (2). Of note, recent evidence suggests that, in addition to a lysosomal destination, a significant fraction of internalized epidermal growth factor (3), serum immunoglobulin A (4), mannosylated proteins (5), and asialotransferrin (6) escape lysosomal destruction and eventually reappear as undegraded ligand in the extracellular medium. This phenomenon has been termed diacytosis (6) or ligand recycling.

These alternative, nonlysosomal pathways of ligand and receptor movement must be further dissected to

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allow selective targeting of ligands to intracellular compartments. A critical, quantitative examination of the characteristics of any ligand movement requires a well defined system of ligand-receptor interaction in a uniform cell population. The transfer of galactose-terminal glycoproteins into the cloned human hepatoma cell line, Hep G2, is a well characterized system for the examination of the kinetics of events during receptor-mediated endocytosis (7). The present study was devised to characterize the single cycle kinetics of galactose-terminal glycoprotein movement following binding to the asialoglycoprotein receptor and internalization in a cloned human hepatoma cell line. Specific pharmacologic agents were utilized as probes to inhibit steps in the pathway of receptor-mediated endocytosis and thus define the characteristics of ligand and receptor movement. Our findings demonstrate that a major pathway of ligand movement involves binding to ASGP-R,[2] internalization, and return to the cell surface as both free ligand and ligand-receptor complexes.

EXPERIMENTAL PROCEDURES

Materials. Human orosomucoid was obtained from the American Red Cross. ASOR was prepared by acid hydrolysis as previously described (8). The degree of desialylation was assayed according to the method of Warren (9). Cytochrome *c* (Sigma) was galactosylated according to the method of Lee *et al.* (10); cyanomethyl 1-thio galactopyranoside was obtained from E-Y Associates. The degree of galactosylation of cytochrome *c* was assayed by the method of Dubois *et al.* (11). $Na^{125}I$ was obtained from the Amersham Corporation. Ligands were iodinated with chloramine-T as previously described (8). Specific radioactivities ranged from 500 to 3000 cpm/ng of protein. ^{125}I-iodinated proteins were analyzed by SDS-PAGE (12). Eagle's minimal essential medium and fetal bovine serum were from Gibco. Saponin, *p*-nitrophenyl-*N*-acetylglucosamine, primaquine bisphosphate, Triton X-100, and sodium azide were obtained from Sigma. ^{14}C-protein standards and [^{35}S]methionine were purchased from the Amersham Corporation. IgG-Sorb was obtained from the New England Enzyme Center. All reagents were of the highest purity available.

Tissue culture. The maintenance of the a16 clone of human hepatoma cell line Hep G2 has been detailed previously (13). Cells were seeded onto 35-mm culture dishes (Costar) 4 days prior to each experiment, refed with fresh media 1 day prior to use, and utilized at 90–95% confluence (approximately 1×10^6 cells/dish). During all experimental procedures, cells maintained greater than 95% viability as determined by trypan blue dye exclusion.

Binding, internalization, and exocytosis. Cells were washed at 4° with Dulbecco's PBS containing 1.7 mM Ca^{2+} (PBS/C) and incubated for 2 hr at 4° with 50 mM ligand in 1 ml of binding medium (Eagle's minimal essential medium, 0.1 mg/ml cytochrome *c*, and 10 mM HEPES, pH 7.3). Following saturation binding, monolayers were washed free of unbound ligand in PBS/C at 4° (14). The cells were then allowed to internalize ligand for up to 5 min by incubation in prewarmed binding medium at 37° with or without metabolic inhibitors as indicated. Subsequent to internalization, monolayers were immediately chilled by immersion in PBS/C at 4°. Ligand remaining on the cell surface was removed by exposure to phosphate-buffered saline with no added calcium but containing 10 mM EDTA (PBS/EDTA), pH 5.0 for 5 min at 4°. Cells were then incubated in 1 ml of binding medium with or without 50 nM unlabeled ASOR and metabolic inhibitors as indicated at 37°. After incubation, exocytosis medium was collected at the appropriate time and radioactivity was determined in chilled 10% trichloroacetic acid-precipitable and soluble fractions. Cells were solubilized in 1 ml of 1 M NaOH and subsequently assayed for total protein as previously described (14) and radioactivity (Packard gamma counter). Specific uptake (or exocytosis) was defined as total uptake minus nonspecific uptake determined in the presence of 5 μM unlabeled ASOR (14).

Cell permeabilization with saponin. Determination of intracellular free and membrane-bound ligand was accomplished after treatment with the nonionic detergent saponin (15, 16). Cell monolayers were washed in PBS/C at 4° and incubated for 2 hr at 4° with 50 nM ^{125}I-ASOR or 200 nM ^{125}I-Gal-cytochrome *c* in 1 ml of binding medium. Monolayers were washed free of unbound ligand in PBS/C at 4° and then allowed to internalize ligand for 5 min by incubation in prewarmed binding medium at 37°. Monolayers were immediately immersed in PBS/C at 4°, followed by removal of cell surface ligand by exposure to PBS/EDTA for 5 min at 4°. Monolayers were then exposed to 1 ml of binding medium containing 0 to 0.5 g/dl saponin for 0 to 40 min at 4°. Medium was collected and specific TCA-precipitable radioactivity was determined (free intracellular ligand). Cells were rinsed in PBS/C at 4° and solubilized in 1 ml 1 M NaOH, and specific saponin-resistant radioactivity was determined. As seen in Fig. 1 (upper and lower

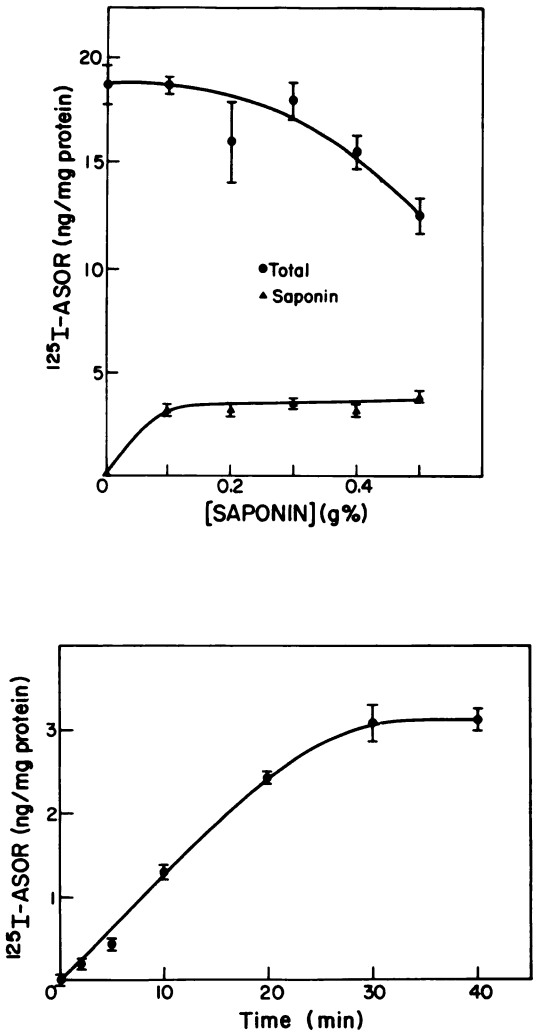

FIG. 1. *Saponin effects in HepG2 cells*

Upper panel, effect of saponin concentration on release of intracellular ^{125}I-ASOR. Lower panel, time course of saponin permeabilization of HepG2 cell monolayers. See text for details.

[2] The abbreviations used are: ASGP-R, asialoglycoprotein receptor; ASOR, asialoorosomucoid; PBS, phosphate-buffered saline; GalNAc, *N*-acetylgalactosamine; TCA, trichloroacetic acid; SDS-PAGE, sodium dodecyl sulfate-polycrylamide gel electrophoresis; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesufonic acid.

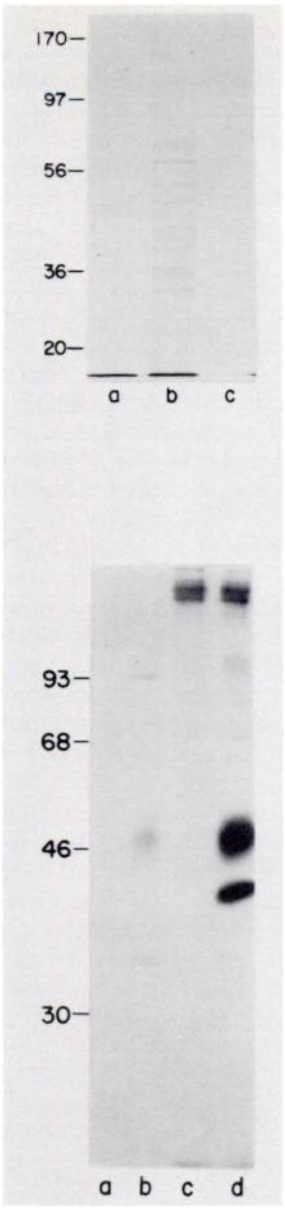

FIG. 2. *Electrophoretic results*

Upper panel, SDS-PAGE of exocytosis medium (*a*), saponin medium (*b*), and cell lysate (*c*). See text for details. Molecular weight markers are displayed in kilodaltons. Lower panel, saponin-induced release of biosynthetically labeled ASGP-R. See text for details. Lane *a*, saponin medium preimmune; lane *b*, saponin medium, anti-ASGP-R antibody; lane *c*, cell lysate preimmune; lane *d*, cell lysate, anti-ASGP-R antibody. Lane *d* demonstrates the 40,000-Da precursor and 46,000-Da mature forms of ASGP-R. Lane *b* represents less than 5% of the total receptor radioactivity in lane *d*.

panels), 0.1 g/dl saponin releases maximal free intracellular ^{125}I-ASOR after 30 min at 4°.

In order to determine the extent of release of cellular proteins, HepG2 monolayers were exposed to 1 ml of binding medium containing 0.1 g/dl saponin for 30 min at 4°. The medium was collected and the cells were solubilized in 1% Triton X-100. Aliquots of saponin medium and solubilized cell lysate were analyzed by 7.5% SDS-PAGE (12) and stained with Coomassie blue. As seen in Fig. 2 (upper panel), cell proteins in excess of 100 kD are liberated into the medium by such conditions.

To determine the extent of solubilization by saponin of integral membrane proteins, ASGP-R was biosynthetically labeled with [^{35}S] methionine and immunoprecipitated from both saponin-permeabilized monolayers and medium. As previously described (13), HepG2 cell monolayers were exposed to methionine-deficient medium for 15 min at 37° and then pulsed with 200 μCi of [^{35}S]methionine in 1 ml of methionine-deficient medium for 2 hr at 37°. Monolayers were washed in PBS/C at 4° and permeabilized with 1 ml of binding medium containing 0.1 g/dl saponin for 40 min at 4°. Medium and cells were then solubilized in 1% Triton X-100 in PBS containing 1 mM phenylmethylsulfonyl fluoride and immunoprecipitated overnight at 4° with 15 μg of preimmune or anti-ASGP-R affinity-purified antibody. The immunoprecipitates were isolated with 50 μl of 10% formalin-fixed *Staphylococcus*-bearing protein A (1 hr at 4°) and analyzed by 10% SDS-PAGE (12). Gels were prepared for fluorography with Autofluor, dried, and exposed to prefogged Kodak SB5 film. Densitometry was performed utilizing a Helena Quick-Scan scanning densitometer. Figure 2 (lower panel) reveals that permeabilization with saponin as described solubilizes much less than 5% of total cell ASGP-R; 96% of hexosaminidase A was released by saponin pretreatment, as assessed by the method of Hall *et al.* (17).

To determine intracelluar free and membrane-bound ligand after binding, internalization, and exocytosis, cells were chilled by immersion in PBS/C at 4° and then exposed to 1 ml of binding medium containing 0.1 g/dl saponin for 30 min at 4°. Medium was collected and specific TCA-precipitable radioactivity was determined (free intracellular ligand). Cell monolayers were then exposed to 1 ml of PBS/EDTA, pH 5.0 for 5 min at 4°. Medium was collected and specific TCA-precipitable radioactivity was determined (membrane-bound intracellular ligand). Cells were solubilized in 1 ml of NaOH and subsequently assayed for specific radioactivity and total protein as above.

RESULTS

In addition to the expected pathway of lysosomal degradation, single cohort kinetics indicated that a large fraction of internalized ^{125}I-ASOR returned free and undegraded to the surrounding medium.

Exocytosis of internalized ^{125}I-ASOR. Figure 3 (upper panel) demonstrates the time course of reappearance of specific TCA-precipitable radioactivity in the exocytosis medium at 37°. Previously, we established that, following saturation of Hep G2 ASGP surface receptors with ^{125}I-ASOR, 5 min of internalization at 37° and 5 min of "stripping" with PBS/EDTA, pH 5.0 (4°), much less than 5% of total specifically bound ligand remains on the cell surface (14). In contrast, reappearance of TCA-precipitable radioactivity in the exocytosis medium approaches an apparent maximum of 27.8% of the total specific cell-associated radioactivity. The kinetics of exocytosis are represented in Fig. 3 (lower panel). A semilogarithmic plot of [1 minus (exocytosis at time t divided by maximal exocytosis)] versus time yields a straight line, thus consistent with a pseudo-first order process. The half-time of exocytosis is 84 min, and $k = 0.008$ min^{-1}.

The nature of the TCA-precipitable radioactivity returning to the exocytosis medium was first examined by gel filtration chromatography. After surface binding, internalization, and surface "stripping," and 2 hr of incubation at 37°, greater than 70% of cell-associated radioactivity co-migrated with phenol red and [^{125}I]iodotyrosine, as expected for the migration of products of lysosomal degradation (data not shown). In contrast, 30% of the ^{125}I radioactivity in the exocytosis medium 2 hr after internalization co-migrates with ^{125}I-ASOR.

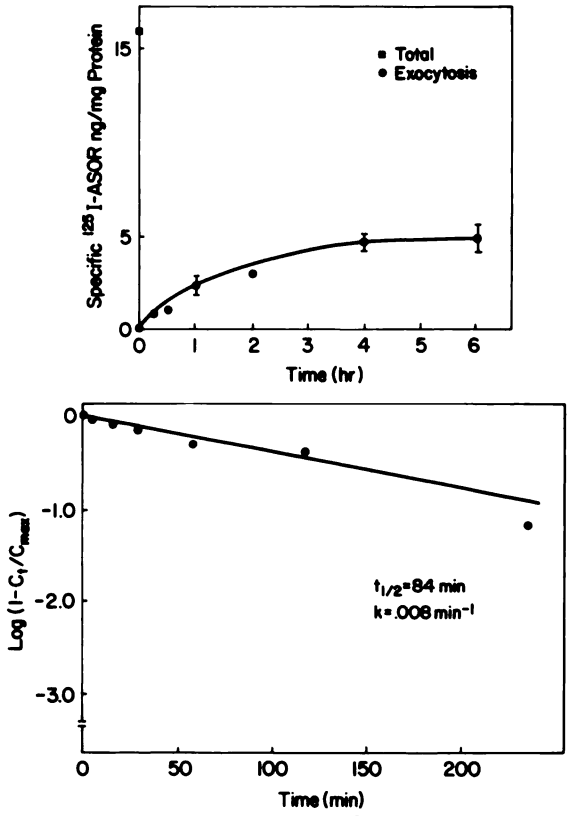

FIG. 3. *Exocytosis of internalized* ^{125}I-ASOR

Following saturation binding at 4° in the presence of 50 nM ^{125}I-ASOR, cell monolayers (1 × 10^6 cells) were washed in PBS/C at 4° and allowed to internalize at 37° for 5 min. Noninternalized, surface-bound ^{125}I-ASOR was removed by rinsing with PBS/EDTA, pH 5.0 for 5 min at 4°. Exocytosis in the presence of 50 nM unlabeled ASOR was allowed to proceed for 15 min to 6 hr at 37°. TCA-precipitable radioactivity in the exocytosis medium was determined and normalized for cell protein and is expressed as specific nanograms of ^{125}I-ASOR per mg of cell protein (upper panel). Total internalized ^{125}I-ASOR represents the sum of specific radioactivity in the medium plus specific cell-associated radioactivity. Specific uptake was defined as ^{125}I-ASOR uptake alone minus ^{125}I-ASOR uptake in the presence of a 100-fold excess of unlabled ASOR. Each point represents the mean of three to four determinations ± standard error. The lower panel represents the kinetics of ^{125}I-ASOR exocytosis transformed according to the relation log $(1 - C_t/C_{max})$ where C_t represents the amount of ASOR exocytosis at time t and C_{min} represents maximum ASOR exocytosis over 6 hr.

Similarly, the TCA-precipitable radioactivity from the exocytosis medium co-migrates with native ^{125}I-ASOR on SDS-10% PAGE (data not shown). Of importance, the percentage of radioactivity that co-migrates with ^{125}I-ASOR accounts for greater than 90% of the TCA-precipitable radioactivity in the exocytosis medium.

The exocytosis of internalized ^{125}I-ASOR is proportional both to initial time of internalization of receptor-ligand complexes and to per cent surface ASGP receptor saturation. Thus, the fractional exocytosis of ^{125}I-ASOR is approximately 25% of initially internalized ^{125}I-ASOR at all values of internalization time or per cent surface ASGP receptor saturation (data not shown).

Return of ligand-receptor complexes to the cell surface. In addition to free ligand returning to the extracellular medium, there was evidence of return of intact receptor-ligand complexes to the cell surface. Following internalization and incubation at 37° for up to 8 min, cells were rapidly chilled to 4° and exposed to conditions promoting the immediate dissociation of ligand from the cell surface at 4°. As seen in Fig. 4, within 5 min of maximum internalization at 37°, increasing amounts of TCA-precipitable radioactivity returns to the cell surface. This radioactivity is sensitive to the dissociating agent GalNAc at 4°, consistent with ASGP-receptor bound ^{125}I-ASOR (18). Of note, the effects of 100 mM GalNAc were identical to those obtained in experiments utilizing PBS/EDTA as the dissociating agent at 4° (data not shown).

Exocytosis of ^{125}*I-ASOR during enhanced ligand-receptor dissociation.* In order to quantify the cumulative amount of ligand-receptor complexes returning to the cell surface, exocytosis of ^{125}I-ASOR was examined at 37° in the presence of isotonic binding media containing 100 mM GalNAc. These conditions promote the rapid dissociation of ^{125}I-ASOR from ASGP receptors (18). Figure 5 (upper panel) illustrates the time course of reappearance of specific TCA-precipitable radioactivity in the exocytosis medium at 37°. In contrast to exocytosis in the absence of GalNAc, reappearance of TCA-precipitable radioactivity in the medium approaches a higher maximum value, i.e., 45.6% of internalized ^{125}I-ASOR. Figure 5 (lower panel) verifies that the pseudo-first order nature of exocytosis still exists but that the process is much more rapid, $t_{1/2} = 24$ min, $k = 0.029$ min^{-1}. Of note, reversal of the order of exposure of cells to GalNAc and ligand (i.e., initial 37° incubation in the presence of isotonic binding medium containing 100 mM GalNAc for 5 min, followed by saturation binding of ^{125}I-ASOR at 4°, internalization for 5 min at 37°, and exocytosis in medium devoid of GalNAc) did not increase exocytosis

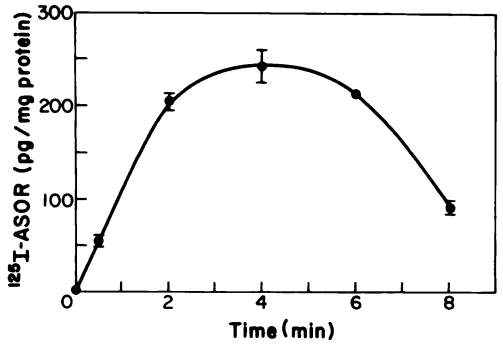

FIG. 4. *GalNAc-sensitive cell-associated radioactivity following internalization of* ^{125}*I-ASOR*

Following saturation binding at 4° in the presence of 50 nM ^{125}I-ASOR, cell monolayers (1 × 10^6 cells) were washed in PBS/C at 4° to remove unbound ligand and thereafter incubated at 37° in prewarmed binding media for 5 min. Noninternalized, surface-bound ^{125}I-ASOR was removed by rinsing with isotonic binding medium containing 100 mM GalNAc for 5 min at 4°. Exocytosis in binding medium containing 50 nM unlabeled ASOR proceeded for up to 8 min at 37°. Medium was collected and monolayers were chilled to 4° at the appropriate time. Monolayers were then washed with 1 ml of isotonic binding medium containing 100 mM GalNAc for 5 min at 4°. Specific TCA-precipitable radioctivity was determined in the wash medium and is expressed as picograms of ^{125}I-ASOR/mg of cell protein. Each point represents the mean ± standard error, $n = 3$; total internalized ^{125}I-ASOR = 10 ng/mg of protein.

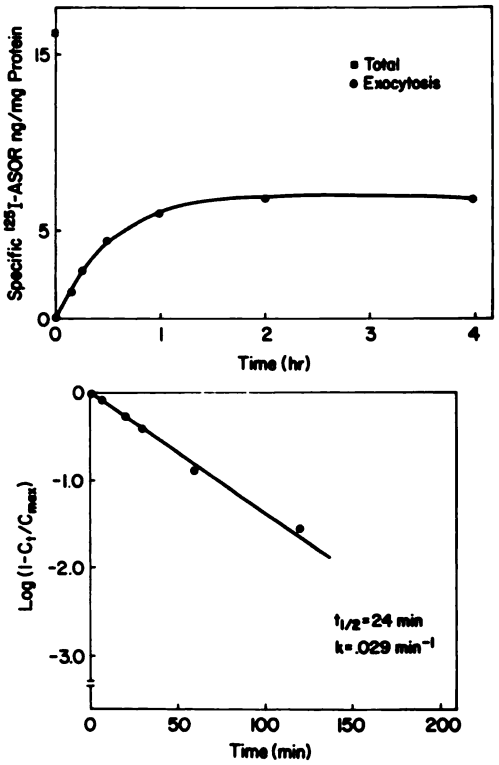

FIG. 5. *Exocytosis of internalized ^{125}I-ASOR in the presence of GalNAc*

Following saturation binding at 4° in the presence of 50 nM ^{125}I-ASOR, monolayers were washed in PBS/C at 4° and allowed to internalize at 37° for 5 min. Exocytosis in the presence of 50 nM unlabeled ASOR and 100 mM GalNAc proceeded from 15 min to 4 hr at 37°. Specific TCA-precipitable radioactivity in the exocytosis medium (upper panel) was counted and expressed as in Fig. 3. Each point represents the mean ± standard error of three to four determinations. The lower panel depicts the kinetics of ^{125}I-ASOR exocytosis in the presence of 100 mM GalNAc. The data are derived from the upper panel as in Fig. 3.

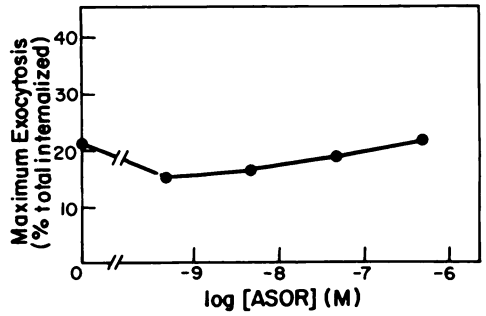

FIG. 6. *Exocytosis of internalized ^{125}I-ASOR versus unlabeled ASOR concentration*

Cell monolayers were saturated with ^{125}I-ASOR at 4°, internalized for 5 min at 37°, and stripped of surface-bound ligand with PBS/EDTA at 4° as in Fig. 3. Exocytosis proceeded for 2 hr at 37° in the presence of 0 to 10^{-6} M unlabeled ASOR. Specific TCA-precipitable radioactivity in the exocytosis medium and cell-associated radioactivity were counted as in Fig. 3 and expressed as per cent total internalized ^{125}I-ASOR. Each point represents the mean of three to four determinations.

of ^{125}I-ASOR compared to controls (data not shown). These data suggest that the mechanism of action of GalNAc which results in enhanced ^{125}I-ASOR exocytosis predominantly involves dissociation of ligand-receptor complexes at the cell surface. Furthermore, in contrast to exocytosis of ASOR in the absence of GalNAc, minimal additional ligand appeared in the exocytosis medium beyond 90 min of incubation. Finally, products of ^{125}I-ASOR degradation released in the exocytosis medium were reduced by 33% in the presence of GalNAc (data not shown).

Effect of unlabeled ASOR on exocytosis of ^{125}I-ASOR. Since internalized ligand returned to the surface still bound to ASGP-R and to the medium as free ligand, the effect of free unlabeled ASOR on the exocytosis of ^{125}I-ASOR was determined. As seen in Fig. 6, decreasing the concentration of ASOR in the exocytosis medium to zero results in no significant reduction in exocytosis of ^{125}I-ASOR. Thus, rebinding of free exocytosed ^{125}I-ASOR to surface ASGP-R is improbable. Furthermore, short term regulation of the extent of exocytosis by ambient ligand concentration seems unlikely.

Intracellular dissociation of ^{125}I-ASOR and ASGP-R. In order to explore the relationship between the release of intracellular ^{125}I-ASOR from ASPG-R and appearance of free ^{125}I-ASOR in the exocytosis medium, the time course of ^{125}I-ASOR/ASGP-R dissociation was determined in saponin-permeabilized cell monolayers. Figure 7 (upper panel) demonstrates that after 5 min of internalization, 25% of ^{125}I-ASOR has rapidly dissociated from ASGP-R, similar to findings in isolated rat hepatocytes (16). A slow phase of dissociation of membrane-bound ^{125}I-ASOR ensues over the next 4 hr. Simultaneously, free intracellular ^{125}I-ASOR initially increases and then decreases progressively over the following 4 hr. Of note, the appearance of TCA-precipitable radioactivity in the exocytosis medium (1.2 ng of ^{125}I-ASOR/mg of protein) is exceeded by the decrease in membrane-bound ^{125}I-ASOR over 4 hr (3.1 ng of ^{125}I-ASOR/mg of protein). Thus, whether reappearance of free ^{125}I-ASOR in the exocytosis medium results from intracellular versus cell surface dissociation of ligand from receptor remained unanswered.

Characterization of galactose-cytochrome c. In order to further investigate the possible sites of dissociation of ligand from the ASGP-R, a new galactose-terminal ligand was synthesized. ^{125}I-Gal-cytochrome *c* exhibits saturable, specific binding to ASGP receptors on the surface of Hep G2 cells. However, the affinity of ^{125}I-ASOR [$K_a = 2 \times 10^{-9}$ M (14)] exceeds that for ^{125}I-Gal-cytochrome *c* ($K_a = 2 \times 10^{-7}$ M, Fig. 8, upper panel). ^{125}I-Gal-cytochrome *c* averaged 2 mol of galactose per mol of cytochrome *c*, and migrated predominantly as a 13,000-Da polypeptide when analyzed by 10% SDS-PAGE (data not shown). Of importance, ^{125}I-Gal-cytochrome *c* demonstrated more rapid spontaneous dissociation kinetics at 37° versus ^{125}I-ASOR (see Fig. 8, lower panel; $t_{1/2} = 8$ versus 25 min, respectively).

Exocytosis of internalized ^{125}I-Gal-cytochrome c. Figure 9 (upper panel) illustrates the exocytosis of internalized ^{125}I-Gal-cytochrome *c* at 37° versus time. Reappearance of TCA-precipitable radioactivity attains a maximum of 40% of specific internalized ^{125}I-Gal-cytochrome *c*. Kinetics of exocytosis of ^{125}I-Gal-cytochrome *c* demonstrate

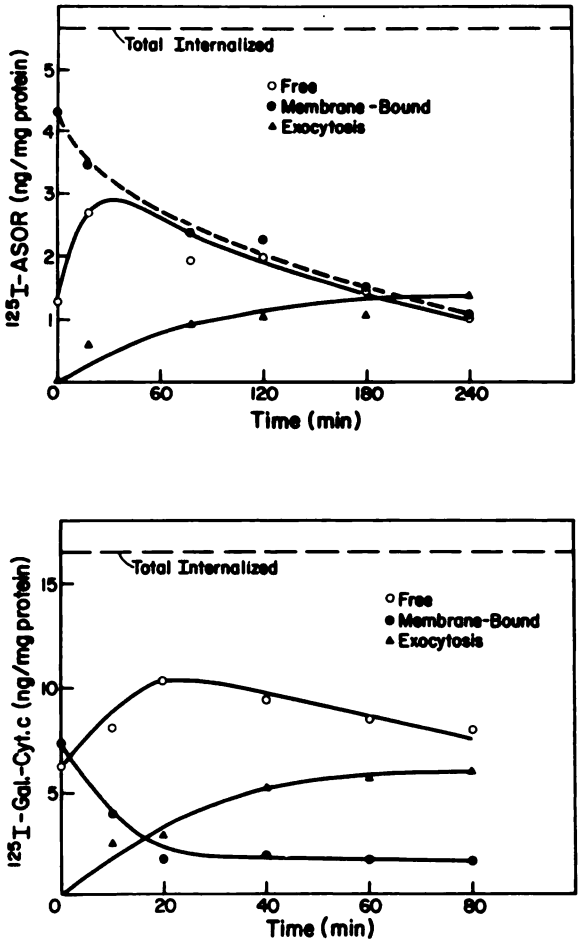

FIG. 7. *Intracellular ligand-receptor dissociation*

Upper panel, intracellular free and membrane-bound ^{125}I-ASOR following internalization. Following saturation binding at 4° in the presence of 50 nM ^{125}I-ASOR, monolayers were washed in PBS/C at 4° and allowed to internalize at 37° for 5 min. Surface ^{125}I-ASOR was removed by rinsing with PBS/EDTA for 5 min at 4°. Exocytosis in the presence of 50 nM unlabeled ASOR proceeded from 0 to 4 hr at 37°. Media were collected and cells were permeabilized with 1 ml of binding medium containing 0.1 g/dl saponin for 30 min at 4°. Media were again collected and the saponin-permeabilized cells were rinsed with PBS/EDTA for 5 min at 4°. Specific TCA-precipitable radioactivity in the exocytosis medium, saponin medium (free intracellular ^{125}I-ASOR), and PBS/EDTA medium (membrane-bound ^{125}I-ASOR) was determined and expressed as in Fig. 3. Each point represents the mean of three to four determinations. Lower panel, intracellular free and membrane-bound ^{125}I-Gal-cytochrome *c* following internalization. Following saturation binding at 4° in the presence of 200 nM ^{125}I-Gal-cytochrome *c*, monolayers were treated as detailed above (upper panel). Specific TCA-precipitable radioactivity in the exocytosis medium, saponin medium (free intracellular ^{125}I-Gal-cytochrome *c*), and PBS/EDTA medium (membrane-bound ^{125}I-Gal-cytochrome *c*) was determined and expressed as in Fig. 3. Each point represents the mean of three determinations.

a half-time of 29 min and $k = 0.024$ min^{-1} [Fig. 9 (lower panel)]. Data are consistent with a single compartment, pseudo-first order process. Thus, a galactose-terminal glycoprotein which spontaneously dissociates from the ASGP-R at a more rapid rate demonstrates more rapid and extensive exocytosis when compared to exocytosis of ^{125}I-ASOR.

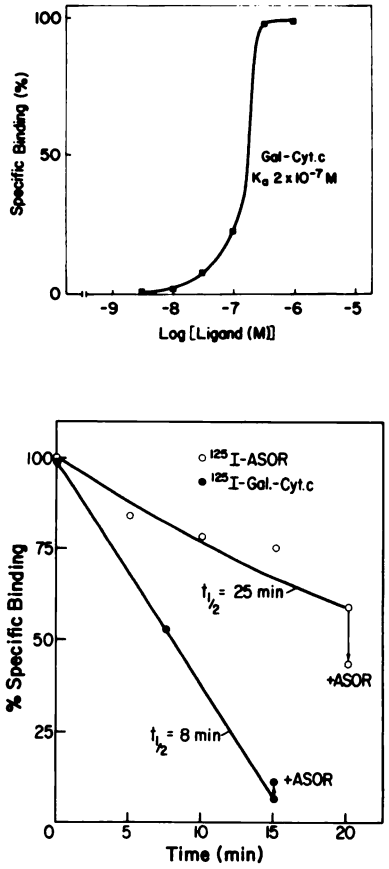

FIG. 8. *Characterization of ^{125}I-Gal-cytochrome c*

^{125}I-Gal-cytochrome *c* was prepared as described in the text. Monolayers were exposed to concentrations of ligand from 10^{-9} to 10^{-6} M and allowed to bind at 4° for 2 hr. Cells were washed in PBS/C at 4°, solubilized in 1 N NaOH, and cell-associated radioactivity was determined. Specific binding of ligand was defined as binding of ligand minus binding of ligand in the presence of a 100-fold molar excess of unlabeled ASOR (upper panel). Each point represents the mean of three to four determinations and is expressed as per cent maximal specific binding (54.6 ng/mg of protein). To determine rates of dissociation at 37°, monolayers were exposed to 10 mM sodium azide in 1 ml of binding medium for 15 min at 37°. Monolayers were then exposed to 1 ml of binding medium with 10 mM sodium azide containing either 50 nM ^{125}I-ASOR or 200 nM ^{125}I-Gal-cytochrome *c* for 15 min at 37°. Monolayers were rinsed in PBS/C at 4° and incubated up to 20 min in 1 ml of prewarmed binding medium at 37°. Monolayers were rinsed in PBS/C at 4° at the appropriate time and remaining specific cell-associated radioactivity was determined. Results are expressed as per cent specific binding versus time (lower panel). Each point represents the mean of three determinations; 50 nM unlabeled ASOR was included in the dissociation medium of experiments as noted.

Intracellular dissociation of ^{125}I-Gal-cytochrome c and ASGP-R. The intracellular dissociation of ^{125}I-ASOR could now be contrasted with that of ^{125}I-Gal-cytochrome *c*, a ligand demonstrating more rapid spontaneous dissociation kinetics. Figure 7 (lower panel) demonstrates that, after 5 min of internalization, 60% of ^{125}I-Gal-cytochrome *c* has rapidly dissociated from ASGP-R. The slow phase of dissociation of membrane-bound ^{125}I-Gal-cytochrome *c* is more brief and rapid than that observed for ^{125}I-ASOR. Of importance, the appearance of TCA-precipitable radioactivity in the exocytosis medium between 20 and 80 min of exocytosis (3.5 ng of ^{125}I-Gal-

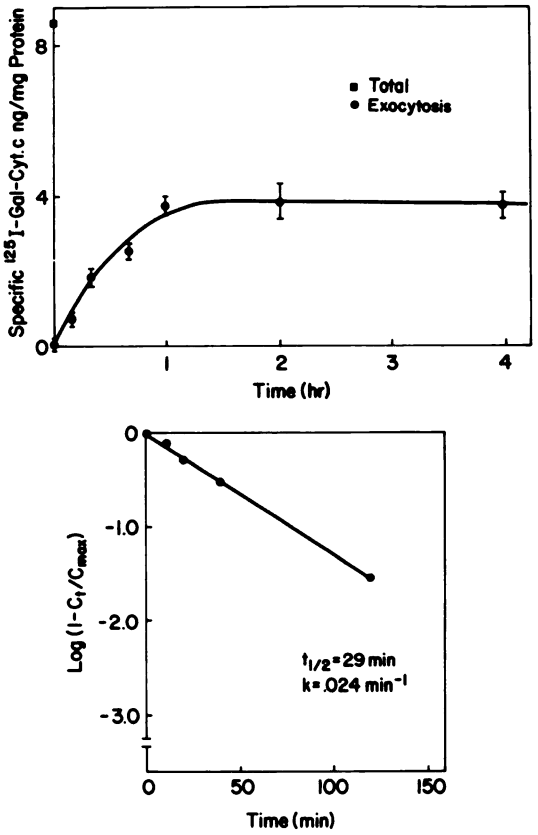

FIG. 9. *Exocytosis of internalized ^{125}I-Gal-cytochrome c*

Following saturation binding at 4° in the presence of 200 nM ^{125}*I-Gal-cytochrome c*, monolayers were washed in PBS/C at 4° and allowed to internalize at 37° for 5 min. Surface-bound ^{125}I-Gal-cytochrome *c* was removed by rinsing with PBS/EDTA, pH 5.0 for 5 min at 4°. Exocytosis in the presence of 50 nM unlabeled ASOR proceeded from 15 min to 4 hr at 37° (upper panel). TCA-precipitable radioactivity in the exocytosis medium was counted, normalized for cell protein, and expressed as specific nanograms of ^{125}I-Gal-cytochrome *c* per mg of cell protein. Each point represents the mean ± standard error of three to four determinations. Specific uptake was defined as ^{125}I-Gal-cytochrome *c* uptake alone minus ^{125}I-Gal-cytochrome *c* uptake in the presence of a 100-fold excess of unlabeled ASOR. The lower panel depicts the kinetics of ^{125}I-Gal-cytochrome *c* exocytosis, with data derived from the upper panel as explained in Fig. 3.

cytochrome *c*/mg of protein) greatly exceeds the decrease of membrane-bound ^{125}I-Gal-cytochrome *c* (0 ng of ^{125}I-Gal-cytochrome *c*/mg of protein) which has reached its absolute minimum by 20 min of exocytosis. If no new membrane-bound ^{125}I-Gal-cytochrome *c* is added to the system, then intracellular dissociation of ^{125}I-Gal-cytochrome *c* must contribute to the pool detected as free ligand in the exocytosis medium.

Sodium azide sensitivity of exocytosis. In order to reduce the likelihood that ligand-receptor complexes which return to the cell surface are reinternalized, the effects of sodium azide were investigated. Previous experiments from our laboratory have indicated that 10 mM sodium azide eliminates internalization of ^{125}I-ASOR by the ASGP-R after 15 min of preincubation at 37° (7). As seen in Fig. 10 (upper panel), exposure of HepG2 cell monolayers to 10 mM sodium azide results in a normal ^{125}I-ASOR internalization rate for 1 min, followed by a profound decline in the subsequent internalization rate versus control. Following normal internalization for 1 min, the effects of increasing concentrations of sodium azide on exocytosis and degradation of internalized ^{125}I-ASOR were investigated. Figure 10 (middle panel) demonstrates that exposure to increasing sodium azide concentrations resulted in progressive inhibition of exocytosis to 55% of control values. Degradation of internalized ^{125}I-ASOR was more sensitive to sodium azide and decreased to 23% of control values after 1 hr of incubation. Furthermore, Fig. 10 (lower panel) depicts that the degradation of internalized ligand is inhibited by sodium azide in the presence or absence of 100 mM GalNAc in the exocytosis medium. In contast, exocytosis of internalized ^{125}I-ASOR, performed under dissociating conditions in the presence of 100 mM GalNAc, was *not* sensitive to 10 mM sodium azide (95% control value). Since these conditions prevent the internalization of GalNAc and reinternalization of ligand-receptor complexes, enhanced ligand-receptor dissociation must occur primarily at the cell surface. In addition, exocytosis of ligands which rapidly dissociate from the ASGP-R, such as ^{125}I-Gal-cytochrome *c*, is not sensitive to inhibition by 10 mM sodium azide (105% control value; Fig. 10, lower panel). The effects of up to 1 hr of exposure of cell monolayers to 10 mM sodium azide were readily reversible (data not shown).

A decrease in temperature of the exocytosis medium to 4° significantly reduced both degradation and exocytosis of internalized ^{125}I-ASOR to less than 10% of control values (data not shown).

Primaquine sensitivity of exocytosis. In order to explore the possibility that ligand recycling involves an acidic sorting compartment, the effects of primaquine were investigated. Lysosomotropic agents, such as the weak bases chloroquine and primaquine, inhibit endosomal and lysosomal pH (19), inhibit lysosomal protease activity (20), and inhibit ASGP receptor recycling in Hep G2 cell monolayers (21). Figure 11 reveals that the presence of up to 0.1 mM primaquine in the internalization and exocytosis media progressively inhibits appearance in the medium of lysosomal degradation products of internalized ^{125}I-ASOR (to less than 10% control). In contrast, no effect of primaquine on total specific internalization or exocytosis of ^{125}I-ASOR is observed after 2 hr at 37°.

DISCUSSION

Critical to a full understanding of the process of receptor-mediated endocytosis is the elucidation of the intracellular pathways involved in the internalization and sorting of ligand and receptor molecules, as well as their ultimate destinations. Of great interest are recent observations of a novel pathway which leads to ligand recycling and exocytosis of undegraded or modified asialoglycoproteins internalized by the ASGP receptor (6), since the ultimate intracellular fate of galactose-terminal glycoproteins has appeared to be lysosomal destruction. Since the net uptake of ligand by a cell may be a balance between receptor-mediated endocytosis and exocytosis, experimental designs which utilize steady state kinetic analysis cannot discriminate return of undegraded, in-

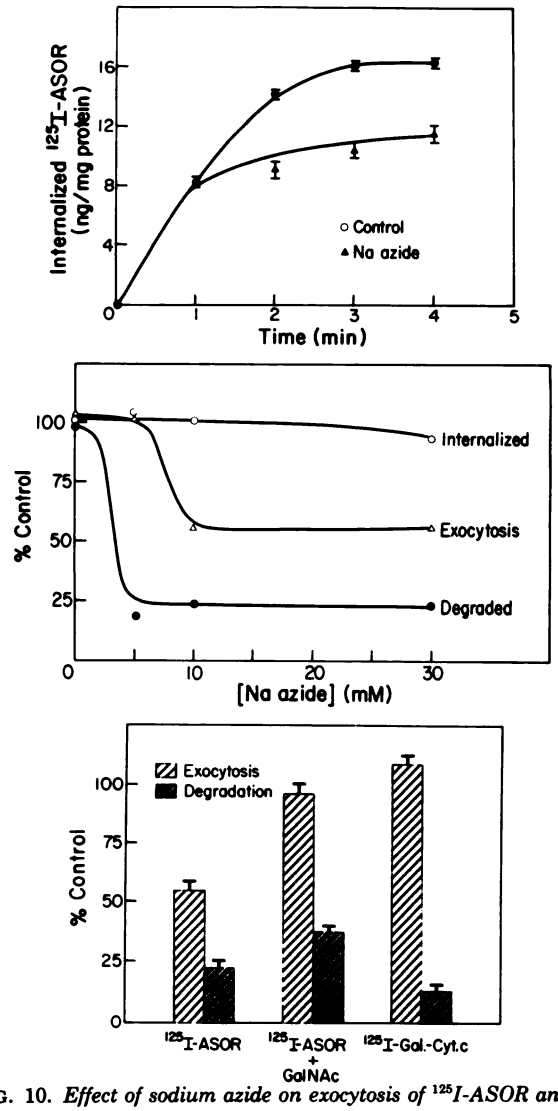

FIG. 10. *Effect of sodium azide on exocytosis of ^{125}I-ASOR and ^{125}I-Gal-cytochrome c*

Following saturation binding with ^{125}I-ASOR (4°), monolayers were exposed to 0 or 10 mM sodium azide during 0 to 4 min of internalization in 1 ml of prewarmed binding medium at 37° (upper panel). Remaining surface-bound ^{125}I-ASOR was removed by rinsing in PBS/EDTA, pH 5.0 for 5 min at 4°. Cells were then solubilized in 1 M NaOH; specific radioactivity was determined and expressed as specific nanograms of ^{125}I-ASOR per mg of cell protein. Each point represents the mean ± standard error of three determinations. Following saturation binding with ^{125}I-ASOR (4°), cells were allowed 1-min internalization at 37° in 1 ml of prewarmed binding medium containing 0 to 10 mM sodium azide, PBS/EDTA surface "stripping" (4°), and 1 hr of exocytosis in the presence of 50 nM unlabeled ASOR and 10 mM sodium azide (middle panel). Specific TCA-precipitable and acid-soluble radioactivity in the exocytosis medium was counted, normalized for cell protein, and expressed as per cent control values. Each point represents the mean of three determinations. Following saturation binding with ^{125}I-ASOR or ^{125}I-Gal-cytochrome *c* (4°), monolayers were continuously exposed to 10 mM sodium azide during 1 min of internalization (37°), PBS/EDTA, pH 5.0 surface "stripping (4°), and 1-hr exocytosis in the presence of 50 nM unlabeled ASOR or isotonic binding medium containing 100 mM GalNAc (lower panel). Specific TCA-precipitable and acid-soluble radioactivity in the exocytosis medium was counted, normalized for cell protein, and expressed as per cent control values (without sodium azide). Each point represents the mean ± standard error of three determinations.

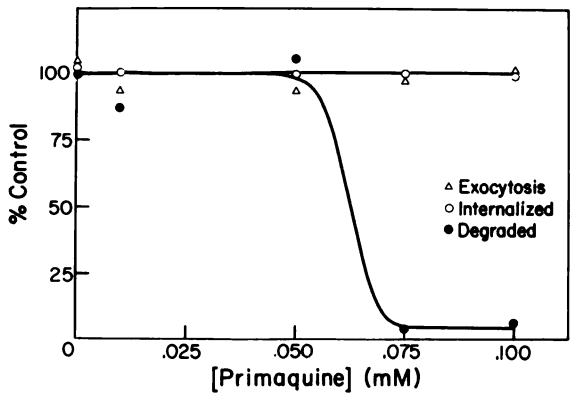

FIG. 11. *Effect of primaquine on exocytosis of ^{125}I-ASOR*

Following saturation binding with ^{125}I-ASOR (4°), monolayers were continuously exposed to 0 to 0.1 mM primaquine during 5 min of internalization (37°), PBS/EDTA, pH 5.0, surface stripping (4°), and 2-hr exocytosis (37°) in the presence of 50 nM unlabeled ASOR. TCA-precipitable and acid-soluble radioactivity in the exocytosis medium was counted, normalized for cell protein, and expressed as percentage of specific ^{125}I-ASOR (total, exocytosis, and acid-soluble) compared to control values (i.e., in monolayers not exposed to primaquine). Each point represents the mean of three determinations (total specific internalized ^{125}I-ASOR = 10.1 ng/mg of protein in control cells).

ternalized ligands to the surrounding medium. In contrast, experiments employing single cohort kinetics, such as the present study, enable one to define the biochemical fate of a cohort of receptor-ligand complexes originating on the cell surface. However, this endeavor requires a homogeneous population of metabolically stable cells with sufficient density of receptors and specific activity of labeled ligands to allow detection of small quantities of ligand molecules in their ultimate compartments.

ASGP-R is localized both to hepatic parenchymal cells and the well differentiated human hepatoma Hep G2 cell line (7). The availability of a stable clone (a16) of cells expressing 250,000 ASGP binding sites per cell and [^{125}I] iodinated natural or synthetic ligands with high specific radioactivity allows the determination of the eventual destination of ligand initially bound to ASGP-R on the cell surface. Furthermore, the well defined kinetics and electron microscopic tracer studies of ASGP receptor-mediated endocytosis in this cell line (22) make it an ideal model system for examination of the pathways utilized by internalized of asialoglycoproteins.

The present study suggests that 28% of a cohort of surface-bound ^{125}I-ASOR eventually returns to the extracellular medium with a $t_{1/2}$ of 84 min in the presence or absence of excess unlabeled ASOR at 37° (Fig. 3). This recycled ligand appears to be of similar electrophoretic mobility as native ligand and is released free into the medium. Our results suggest that single cohort exocytosis of internalized ^{125}I-ASOR as free, undegraded ligand appears to represent a constant proportion (approximately 25%) of the amount of original cell surface ligand. Furthermore, the kinetics of exocytosis are consistent with a first order process (Fig. 3). Thus, a constant fraction of the internal compartment destined for exocytosis reappears in the medium per unit of time.

Free ligand in the exocytosis medium may result from dissociation of surface ligand-receptor complexes or re-

lease of free intracellular ligand. Direct evidence for the return of ^{125}I-ASOR/ASGP-R ligand complexes to the cell surface is provided by the data in Fig. 4. Furthermore, in the presence of conditions which promote dissociation of ^{125}I-ASOR and the ASGP-R (100 mM GalNAc), the cumulative fraction of internalized ligand returning to the surface as ligand-receptor complexes approaches 50% (Fig. 5). Figure 10 (lower panel) suggests that GalNAc promotes ligand-receptor dissociation primarily at the cell surface, since 10 mM sodium azide prevents internalization of GalNAc and has no effect on the enhanced appearance of ^{125}I-ASOR in the exocytosis medium.

In contrast, the data are inconclusive regarding the release of free intracellular ligand following intracellular dissociation of ^{125}I-ASOR from the ASGP-R [Fig. 7 (upper panel)], as the decrease in ASGP-R-bound ^{125}I-ASOR exceeds the increase in free ^{125}I-ASOR returning to the exocytosis medium. In order to increase the likelihood of detecting the release of free intracellular ligand, the intracellular dissociation of a ligand displaying rapid dissociation kinetics (^{125}I-Gal-cytochrome *c*) was examined. Intracellular dissociation of ^{125}I-Gal-cytochrome *c* and return of free ligand to the cell surface must be invoked to explain continued appearance of free ^{125}I-Gal-cytochrome *c* in the exocytosis medium despite minimal remaining membrane-bound intracellular ligand [Fig. 7 (lower panel)]. Thus, ligand appearing in the exocytosis medium may result from release of free intracellular ligand or from dissociation of ligand-receptor complexes at the cell surface, depending upon the ligand examined. Since free unlabeled ASOR has no effect on exocytosis of internalized ^{125}I-ASOR (Fig. 6) and has little effect on spontaneous dissociation of ligand bound to ASGP-R at 37° (Fig. 8, lower panel), then release of free ligand at the cell surface is *not* followed by significant rebinding of ligand to ASGP-R in the absence of unlabeled ASOR.

These findings significantly extend those of Tolleshaug *et al.* (6), who observed diacytosis of approximately 20% of internalized human ^{125}I-asialotransferrin type 3 at 37° in primary rat hepatocyte cultures. This phenomenon occurs *in vivo* as well as *in vitro*, as evidenced by studies demonstrating diacytosis of human ^{125}I-asialotransferrin type 3 following internalization by the liver in the intact rat (23). These previous studies are difficult to interpret since transferrin receptors are likely to coexist with ASGP receptors in endocytotic vesicles. Thus, since transferrin undergoes minimal degradation and returns to the cell surface after delivery of iron to the cell, diacytosis of asialotransferrin molecules might be mediated by either transferrin or ASGP receptors (24). Similarly, Townsend *et al.* (25) recently described in isolated, perfused rat livers that galactose-terminal ligands rapidly return to the cell surface and speculate that this phenomenon might involve return to the cell surface of both free ligand and ligand bound to the ASGP-R (25). Connolly *et al.* (26) as well as Weigel and Oka (27) observed a similar phenomenon in isolated rat hepatocytes. The return of receptor-ligand complexes to the cell surface from an internal, slowly dissociating pool was observed as a normal part of the process of endocytosis.

The present study directly demonstrates the return to the cell surface of free intracellular ligand (^{125}I-Gal-cytochrome *c*) or ligand bound to ASGP-R (^{125}I-ASOR) prior to the appearance of free ligand in the exocytosis medium. Furthermore, *two* pools of ligand returning to the cell surface may now be defined based upon their unique kinetics and sensitivity to inhibitors of ASGP-R recycling.

A short transit pool of ASGP-R-ligand complexes may be defined on the basis of its kinetics of reappearance at the cell surface ($t_{1/2}$ = 24 min; Fig. 5), insensitivity to 10 mM sodium azide [Fig. 10 (lower panel)], and insensitivity to 0.1 mM primaquine (Fig. 11). In addition, the data indicate the presence of a long transit pool of ASGP-R-ligand complexes destined to release free ligand to the exocytosis medium. The kinetics of the long transit pool are slower ($t_{1/2}$ = 24 min; Fig. 3) than the short transit pool. Furthermore, the long transit pool is sensitive to 10 mM sodium azide [Fig. 10 (lower panel)] and insensitive to 0.1 mM primaquine (Fig. 11).

Since brief exposure to 10 mM sodium azide totally inhibits internalization and inhibits ligand exocytosis by 50% (Fig. 10), these data suggest that the long transit pathway may involve multiple cycles of return of ASGP-R-ligand complexes to the cell surface followed by exocytosis or reinternalization. In addition, other distal azide-sensitive steps in various intracellular compartments may be involved. Furthermore, since exposure to 100 mM GalNAc in the exocytosis medium totally converts free ligand in the exocytosis medium to kinetics of the short transit pool ($t_{1/2}$ = 24 min), return of ASGP-R-ligand complexes to the cell surface may precede passage through deeper cellular compartments which lead to long transit pool exocytosis. Exposure to 100 mM GalNAc in the exocytosis medium reduces the ultimate degradation of internalized ^{125}I-ASOR. This observation suggests that the short transit pool of recycling ASGP-R-ligand complexes may also be involved in the subsequent delivery of internalized ligand to the lysosomal compartment.

Neither the short or long transit pool of ^{125}I-ASOR is sensitive to 0.1 mM primaquine (Fig. 11). This observation suggests that the pool of ligand destined for exocytosis does not dissociate from ASGP-R within an acidic sorting compartment. Furthermore, inhibition of ligand degradation in the lysososmal compartment does not result in "spill-over" of internalized ligand into the pool destined for exocytosis following a single round of internalization. Of note, the kinetics of spontaneous dissociation of ligand from ASGP-R at neutral pH, 37° are sufficient to explain the appearance of ligand in the exocytosis medium (Fig. 8).

Our previous kinetic studies of HepG2 cells during steady state uptake of ^{125}I-ASOR may be compared with the kinetic values obtained in the present study. On average, an ASGP receptor binds ligand on the cell surface, internalizes, dissociates from ligand, and recycles back to the cell surface every 15 min at 50 nM ^{125}I-ASOR concentration (7). At much higher ligand concentrations, ASGP receptors may recycle as rapidly as every 8 min (7). In contrast, exocytosis of ligand under nondis-

sociating conditions is a relatively slow process, with half-times ranging from 24 to 84 min. Of note, however, is that these values for ligand recycling represent single cycle kinetics. HepG2 cells are not depleted of ASGP receptors available for endocytosis despite the apparent entry of a large fraction of surface receptors into a slowly dissociating ligand-recycling pool during single cycle experiments. Thus, at steady state, an equilibrium must exist between the rate of disappearance of ASGP-R into a slowly dissociating pool and reappearance of free ASGP-R at the cell surface. Furthermore, receptors do not necessarily remain bound to ligand during the entire cycle of ligand recycling (Fig. 7). Recent *in vivo* evidence in the rat suggests that internalized asialoglycoproteins may undergo partial resialylation and be returned to the circulation (28). The relationship of this phenomenon to the long transit pool of ligand destined for exocytosis in the present study remains to be clarified. Passage of internalized ligands through the Golgi apparatus for minor modification prior to exocytosis may be involved, and can be directly examined only if subtle changes in the sugar composition of exocytosed ligand can be discerned.

Of interest, Tietze *et al.* (5) observed that internalized ligand bound to the mannose receptor of rat alveolar macrophages also returned to the surface as ligand-receptor complexes. This cycling pool demonstrated insensitivity to weak bases and similar kinetics to the short transit pool of ligand described in the present study.

Figure 12 represents a hypothetical scheme of the pathways involved in the internalization, ligand recycling, receptor recycling, and degradation of galactose-terminal glycoproteins in the hepatoma cell line HepG2. Interaction of ligand with ASGP receptor at the sinusoidal cell surface is followed by internalization into coated pits and vesicles. Approximately 50% of a single cycle of internalized ligand and surface receptor proceeds to the compartment of uncoupling of receptor and ligand (22). Subsequently, ligand is delivered to lysosomes and degraded while receptor is recycled to the cell surface to undergo further rounds of endocytosis. Of importance, up to 50% of internalized ligand enters a pre-acidic compartment and returns repeatedly to the cell surface. Ligand-receptor dissociation at any point along this pathway may occur and result in free ligand in the exocytosis medium. In addition, an energy-dependent pathway may exist by which internalized asialoglycoproteins are modified and returned to the cell surface.

In summary, up to 28% of internalized ^{125}I-ASOR taken up in a single cycle of ASGP-R-mediated endocytosis can reappear undegraded in the exocytosis medium. This process of ligand recycling follows first order kinetics and represents a constant fraction of initial cell surface or internalized ligand. Both free ligand as well as ligand-receptor complexes may return to the cell surface. Ligand-receptor complexes return repeatedly to the cell surface following a single cycle of internalization. Although an acidic sorting compartment is not required for ligand-receptor dissociation and subsequent exocytosis, energy-dependent passage through other nonlysosomal intracellular organelles may be involved.

Future studies must address the morphologic aspects of the intracellular pathways involved in this novel process of ligand recycling, as well as the mechanism(s) of biochemical modification of internalized asialoglycoproteins prior to exocytosis. Use of similar techniques to

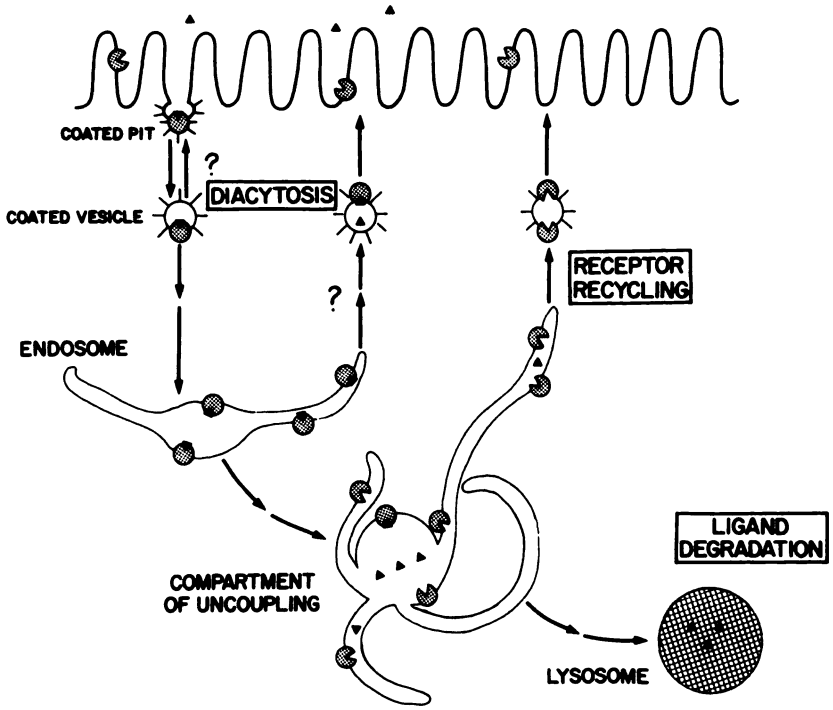

FIG. 12. *Scheme of ligand and receptor pathways during receptor-mediated endocytosis of galactose-terminal glycoproteins*
See text for discussion.

dissect major intracellular pathways taken by ligands following receptor-mediated endocytosis will allow the development of strategies to deliver ligands and pharmacologic agents to specific cellular compartments.

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