Nonpalmitoylated Human Asialoglycoprotein Receptors Recycle Constitutively but Are Defective in Coated Pit-mediated Endocytosis, Dissociation, and Delivery of Ligand to Lysosomes*

Jasper H. N. Yik, Amit Saxena, Janet A. Weigel, and Paul H. Weigel‡

From the Department of Biochemistry & Molecular Biology and The Oklahoma Center for Medical Glycobiology, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma 73190

The hepatic asialoglycoprotein receptor (ASGP-R) internalizes desialylated glycoproteins via the clathrin-coated pit pathway and mediates their delivery to lysosomes for degradation. The human ASGP-R contains two subunits, H1 and H2. Cytoplasmic residues Cys54 and Cys58 in H2 are palmitoylated (Zeng, F.-Y., and Weigel, P. H. (1996) J. Biol. Chem. 271, 32454). In order to study the function(s) of ASGP-R palmitoylation, we mutated these Cys residues to Ser and generated stably transfected SK-Hep-1 cell lines expressing either wild-type or nonpalmitoylated ASGP-Rs. Compared with wild-type ASGP-Rs, palmitoylation-defective ASGP-Rs showed normal ligand binding, intracellular distribution and trafficking patterns, and pH-induced dissociation profiles in vitro. However, continuous ASOR uptake, and the uptake of prebound cell surface ASOR were slower in cells expressing palmitoylation-defective ASGP-Rs than in cells expressing wild-type ASGP-Rs. Unlike native ASGP-Rs in hepatocytes or hepatoma cells, which mediate endocytosis via the clathrin-coated pit pathway and are almost completely inhibited by hypertonic medium, only ~40% of the ASOR uptake in SK-Hep-1 cells expressing wild-type ASGP-Rs was inhibited by hypomolarity. This result suggests the existence of an alternate nonclathrin-mediated internalization pathway, such as transcytosis, for the entry of ASGP-R-ASOR complexes into these cells. In contrast, ASOR uptake mediated by cells expressing palmitoylation-defective ASGP-Rs showed only a marginal difference under hypertonic conditions, indicating that most of the nonpalmitoylated ASGP-Rs were not internalized and processed normally through the clathrin-coated pit pathway. Furthermore, cells expressing wild-type ASGP-Rs were able to degrade the internalized ASOR, whereas ASOR dissociation was impaired and degradation was barely detectable in cells expressing nonpalmitoylated ASGP-Rs. We conclude that palmitoylation of the ASGP-R is required for its efficient endocytosis of ligand by the clathrin-dependent endocytic pathway and, in particular, for the proper dissociation and delivery of ligand to lysosomes.

Numerous studies have demonstrated that the post-translational modification of integral membrane proteins by palmitoylation plays a role in regulating specific protein functions (1–5). For example, CHO cells expressing nonpalmitoylated mutant transferrin receptors show a significant increase in the rate of receptor internalization when compared with the wild-type receptor, indicating that palmitoylation inhibits the rate of transferrin receptor endocytosis (6). Tanaka et al. (7) demonstrated that abolition of palmitoylation reduced the rate of intracellular trafficking of the human thyrotropin receptor, which resulted in delayed surface expression of newly synthesized receptor. Moreover, Blanpain et al. (8) found that failure of the CC-chemokine receptor CCR5 to be palmitoylated hindered its trafficking and thus led to the sequestration of CCR5 in intracellular biosynthetic compartments. Thus, one of the potential mechanisms for regulating intracellular trafficking may be through the covalent attachment of palmitic acid to a protein.

The ASGP-R,1 found in liver parenchymal cells, is an endocytic receptor that can mediate the clearance of injected asialoglycoproteins from the circulation of mammals (reviewed in Refs. 9–19). Native ASGP-Rs in isolated hepatocytes or hepatoma-derived cell lines endocytose asialo- and asialoorosomucoids via the clathrin-dependent pathway. The internalized ligands are ultimately delivered to lysosomes for degradation, while the receptors recycle to the plasma membrane.

Based on a series of studies on ASGP-R-mediated endocytosis, we have previously shown that ASGP-Rs operate in two distinct pathways, called the State 1 and State 2 pathways (reviewed in Refs. 16, 17, 19). Receptors functioning in the State 2 pathway can be distinguished from those in the State 1 pathway based on their sensitivity to modulation by a variety of agents, such as monensin, chloroquine, microtubule drugs, or by ATP depletion (20–22). During the process of endocytosis, only the receptors in the State 2 pathway undergo an I/R cycle that may be regulated by changes in the palmitoylation status of the receptors (23–26). This two-pathway endocytosis system has also been genetically validated by Stockert et al. (27) with the isolation of a trafficking defective mutant cell line, Trf1, derived from HuH-7 cells. Although a functional State 1 pathway is present in the Trf1 cells, they are defective in endocytosis mediated by the State 2 pathway.

The human ASGP-R is composed of two subunits, H1 and H2 (28). The cytoplasmic Cys54 in H1, as well as the analogous Cys54 and the juxtamembrane Cys58 in H2 are palmitoylated (29, 30). In order to elucidate the effects of palmitoylation on...
ASGP-R mediated endocytosis, the Cys residues that are palmitoylated in the H1 and H2 subunits were replaced by Ser using site-directed mutagenesis. The wild-type or mutant subunits were then stably expressed in SK-HeP-1 cells. Here we report that stable cell lines expressing either wild-type or non-palmitoylated ASGP-Rs have a similar cellular distribution pattern of the receptors. However, cells expressing non-palmitoylated ASGP-Rs are defective in ligand uptake, dissociation, and degradation.

**EXPERIMENTAL PROCEDURES**

**Materials—** ASOR was prepared by desialylation of human orosomucoid (Sigma) with neuraminidase as described previously (31). Na°251 (10–20 mCi/µg of iodine) was from Amersham Biosciences. [9,10-3H]palmitate (30–60 Ci/mmol) was purchased from PerkinElmer Life Sciences. 1,2,4,6-Tetrachloro-3,5-diphenylglycouril (Iodogen) and protein assay reagents were from Pierce Chemical Co. Bovine serum albumin was from Intergen Co. 125I-ASOR, iron-saturated human 125I-transferrin, and 125I-anti-H1 and -H2 antibodies were prepared as described previously (32). ASOR conjugated to Alexa Fluor® 488 (fl-ASOR) was prepared using the Alexa Fluor® 488 Protein Labeling Kit from Molecular Probes according to the manufacturer's instructions. The generation and purification of the polyclonal rabbit anti-H1 and goat anti-H2 antibodies have been described previously (32). Sheep anti-rabbit IgG Fab' fragment conjugated to Cy3 was from Sigma. Lysotracker Red DND-99 and donkey anti-goat IgG conjugated to Alexa Fluor 488 were from Molecular Probes. All other reagents were from Sigma unless otherwise noted.

**Buffers and Media—** Buffer 1 contains 150 mM NaCl, 6.7 mM KCl, and 10 mM HEPES, pH 7.4. Hanks balanced salt solution and PBS were made following standard recipes. DMEM and MEM were from Invitrogen. Medium 1 contains MEM supplemented with 2.4 g/liter HEPES, pH 7.4, 100 mg/ml sodium succinate, 75 mg/ml succinic acid, and 0.22 g/liter NaHCO3. Complete medium is DMEM supplemented with 10% fetal bovine serum (Summit Biotechnology), 2 mM L-glutamine, and 100 units/ml penicillin and streptomycin (Invitrogen). Hanks was supplemented with 50 mM sodium succinate, 75 mM succinic acid, 20 mM L-glutamine, and 0.4 M sucrose. Hanks containing 20 mM EGTA was made by treating Hanks with 200 units/ml 1:50-streptokinase and streptodornase (Sigma). Hanks with or without 0.055% (w/v) digitonin, to block clathrin recycling by including 0.4 M sucrose in the medium. The cells were preincubated for 30 min at 37°C to desialylate ASOR and then washed and incubated with DMEM containing 1.5 µg/ml 125I-ASOR for various times at 37°C. In some experiments hyperosmolarity was used to block clathrin recycling by including 0.4 M sucrose in the medium. The cells were preincubated for 30 min at 37°C to desialylate ASOR and then washed and incubated with DMEM containing 1.5 µg/ml 125I-ASOR for various times at 37°C. In some experiments hyperosmolarity was used to block clathrin recycling by including 0.4 M sucrose in the medium. After the desired internalization period, the medium was removed and the cells were washed rapidly on ice, followed by a 10-min incubation with Hanks containing 20 mM EGTA to remove the remaining cell-surface ASOR. The medium was collected and the cells were washed again and lysed. The radioactive recovery in the EGTA wash (cell-surface associated 125I-ASOR) and the cell lysates (internalized 125I-ASOR) were then quantified.

**Internalization of Surface Bound 125I-ASOR—** Stable cell lines were grown in 12-well plates to confluence and serum-starved for 30–60 min. The cells were then incubated on ice for 1 h in Hanks containing 1.5 µg/ml 125I-ASOR. Unbound 125I-ASOR was removed by washing three times with Hanks. The cells were then resuspended in Hanks and incubated at 37°C. In some experiments hyperosmolarity was used to block clathrin recycling by including 0.4 M sucrose in the medium. After the desired internalization period, the medium was removed and the cells were washed rapidly on ice, followed by a 10-min incubation with Hanks containing 20 mM EGTA to remove the remaining cell-surface ASOR. The medium was collected and the cells were washed again and lysed. The radioactive recovery in the EGTA wash (cell-surface associated 125I-ASOR) and the cell lysates (internalized 125I-ASOR) were then quantified.

**Continuous 125I-ASOR Uptake—** The stable cell lines were then confluent and then serum-starved for 30–60 min on ice. The cells were then preincubated for 30 min at 37°C to desialylate ASOR and then washed and incubated with DMEM containing 1.5 µg/ml 125I-ASOR for various times at 37°C. In some experiments hyperosmolarity was used to block clathrin recycling by including 0.4 M sucrose in the medium. The cells were then washed rapidly on ice, washed three times with ice-cold Hanks, and lysed with 0.3 M NaOH. Radioactivity and protein content in the cell lysates were quantified.

**Dissociation of Internalized 125I-ASOR—** Stable cell lines were grown in Lab-Tek® II chamber slides (Nalge Nunc International) to ~70% confluence and then serum-starved for 30 min. The cells were washed, incubated with 1.5 µg/ml fl-ASOR in DMEM, in the presence or absence of 0.4 M sucrose, for 1 h at 37°C. Cells were washed again and then incubated in DMEM containing 150 µg/ml unlabeled ASOR for 30 min. The lysosomal marker Lysotracker Red DND-99 was added at a concentration of 50 nM, and cells were incubated at 37°C for an additional 30 min. After rinsing with PBS, the live cells were examined at room temperature using a Leica TCS confocal microscope equipped with a krypton/argon laser. Digital images of samples that had dual fluorescence staining were captured with Leica TCS NT Version 1.6.587 software. The extent of co-localization of green pixels (fl-ASOR) with red pixels (lysosome) was determined using Adobe Photoshop v5.5 and the image analysis protocol of Setiadi et al. (34).

**Dissociation of Internalized 125I-ASOR—** Cells grown in 12-well plates to confluence were pulse-labeled with 1.5 µg/ml 125I-ASOR in DMEM for 3 min at 37°C and then incubated with a 100-fold excess of unlabeled ASOR for various times, after which the cells were chilled on ice. Remaining cell-associated radioactivity was removed by washing in Hanks containing 20 mM EGTA. The cells were then incubated in 0.5 ml of Hanks, with or without 0.055% (w/v) digitonin, to measure the amount of receptor-bound ligand and the total amount of intracellular ligand (both free and receptor-bound), respectively. After 15 min on ice, the cells were washed and solubilized in 0.3 M NaOH. Quantitation of radioactivity and protein content in the cell lysates was measured.

**pH Sensitivity of ASGP-R ASOR Complexes—** Various stable cell lines were allowed to internalize 125I-ASOR for 30 min at 37°C and chilled rapidly on ice, and unbound ligand was removed by washing three times with ice-cold Buffer 1. The rest of the procedure was performed on ice. Cells were permeabilized with 0.055% (w/v) digitonin in Buffer 1 for 15 min. Cells were then incubated at two different pH values in Buffer 1 for 10 min. The pH of Buffer 1 was adjusted to pH 5.0 to 7.4. The cells were then washed with Buffer 1, and the remaining cell-associated radioactivity was then determined.

**Recycling of Receptor Subunits—** Stable cell lines were grown to confluence in 35 mm dishes and washed with PBS. One ml of prewarmed medium containing 1 mg/ml proteinase-K (Boche Diagnostics Corp.) was added, and the cells were incubated at 37°C. At the desired time, the cells were chilled on ice, scraped, and collected in a microcentrifuge tube, pelleted at 4°C, and washed three times with ice-cold PBS containing 100 µM phenylmethylsulfonyl fluoride. Cells were then solubilized in the latter buffer containing 1% Triton-X 100, 0.1% SDS, and 5 mM EDTA, and the lysates were subjected to reducing SDS-PAGE. The proteins were electrotransferred to nitrocellulose and the H1 and H2 subunits were detected by Western analysis using subunit-specific polyclonal antibodies (32). Images of the Western blots were captured digitally, and the density of the protein bands was quantified using a Fluorchem™ 8000 Imaging System from Alpha Innotech Corp.

**Co-localization of Internalized ASOR and Lysosomes—** Stable cell lines were grown in Lab-Tek® II chamber slides (Nalge Nunc International) to ~70% confluence and then serum-starved for 30 min. The cells were washed, incubated with 1.5 µg/ml fl-ASOR in DMEM, in the presence or absence of 0.4 M sucrose, for 1 h at 37°C. Cells were washed again and then incubated in DMEM containing 150 µg/ml unlabeled ASOR for 30 min. The lysosomal marker Lysotracker Red DND-99 was added at a concentration of 50 nM, and cells were incubated at 37°C for an additional 30 min. After rinsing with PBS, the live cells were examined at room temperature using a Leica TCS confocal microscope equipped with a krypton/argon laser. Digital images of samples that had dual fluorescence staining were captured with Leica TCS NT Version 1.6.587 software. The extent of co-localization of green pixels (fl-ASOR) with red pixels (lysosome) was determined using Adobe Photoshop v5.5 and the image analysis protocol of Setiadi et al. (34).
and remaining cell-associated radioactivity were measured. Nonspecific binding was routinely by including a 100-fold excess of unlabeled ASOR or IgG in the mixes. ASGP-Rs in stable cell lines. Two different cell lines expressing either palmitoylated Cys residues indicated (38). The intracellular 41-kDa precursor polypeptides of H1 (38) remained unaffected by the protease digestion (open arrows), indicating that cells were intact during the experiment. Thus the decrease in the amount of the mature H1 observed at various times was due to degradation by the exogeneous protease of subunits recycling to the cell surface. When the amount of H1 remaining was quantified and compared with the amount recovered at time 0 (Table II), the rates of H1 and H1(C36S) degradation, and thus trafficking from the cell interior to the cell surface, were almost identical with an apparent half-life of ~7 min. The apparent recycling rate of the wild-type H2 was also similar to that of H2(C54S/C58S), with a half-life of ~7 min, when analyzed using the same protocol (not shown). These results indicate that the palmitoylation defect in the receptor subunits does not affect the rate at which H1 or H2 recycles between intracellular compartments and the cell surface.

Continuous Uptake and Degradation of 125I-ASOR Are Slower in Cells Expressing Palmitoylation-defective ASGP-Rs—We next determined the rates of 125I-ASOR uptake in cell lines expressing wild-type or palmitoylation-defective ASGP-Rs. Cells expressing palmitoylation-defective ASGP-Rs internalized ASOR at a reduced rate compared with cells expressing the wild-type ASOR-R (Fig. 4A). This result suggests that abolishing palmitoylation of the ASOR-R inhibited the rate of receptor internalization and ligand accumulation. In control experiments, specific uptake of ASOR was undetectable in SK-Hep-1 cells transfected with the backbone plasmids or in cells transfected with H1 cDNA alone (not shown). The rates of ASOR uptake in both of these control cell lines were comparable to the nonspecific uptake in cells transfected with both ASGP-R subunits.

We also determined the rates of 125I-ASOR degradation in these stable cell lines (Fig. 4B). Wild-type ASGP-Rs mediated the specific degradation of ASOR at a rate at least 7-fold faster than that of the nonpalmitoylated ASGP-Rs, which was only marginally above the nonspecific rate of ASOR degradation. The overall specific ligand processing ability of cells expressing...
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Table I

| ASGP-R type  | 125I-ASOR binding | 125I-IgG binding |
|--------------|-------------------|-----------------|
|              | Surface | Total | Anti-H1 | Anti-H2 |
| H1 + H2     | 17.3 ± 0.3 | 63.0 ± 3.9 | 33.5 ± 11.3 | 75.4 ± 3.8 |
| H1(C36S) + H2(C54S/C58S) | 12.7 ± 1.8 | 56.7 ± 1.2 | 30.2 ± 8.9 | 82.0 ± 19.5 |
| H1(C36S) + H2 | 18.2 ± 3.6 | 66.1 ± 0.9 | 32.0 ± 1.5 | 84.1 ± 15.4 |
| H1 + H2(C54S/C58S) | 12.1 ± 0.3 | 47.6 ± 1.2 | 35.0 ± 5.4 | 63.0 ± 6.7 |

Table II

| Subunit                  | Time (minutes) | Total protein remaining (% time-zero) |
|--------------------------|---------------|--------------------------------------|
| H1                       | 5             | 69.0 ± 5.7 26.6 ± 9.9 5.9 ± 1.6       |
| H1(C36S)                 | 10            | 57.0 ± 7.8 24.7 ± 15.9 6.8 ± 0.1       |
| H1(C36S) + H2(C54S/C58S) | 15            | 51.0 ± 6.8 23.8 ± 14.9 3.8 ± 0.8       |

The densities of the H1 bands from four experiments, performed as in Fig. 3, were quantified, and the percentage of H1 subunit loss at each time point, relative to the amount of H1 protein recovered at time 0 (100%), was determined. Results are the mean ± S.D. (n = 4).

The differences in the rates of ASOR uptake and degradation in these stable cell lines could be explained by a general defect in the coated pit endocytic pathway in an individual cell line. To test this possibility, we measured the endogenous rate of transferrin uptake and its sensitivity to hyperosmolarity as a control (Fig. 5). The rates of transferrin uptake were essentially identical in both cell lines and the accumulation of transferrin plateaued in both cases after ~1 h (not shown). The percent inhibition of transferrin uptake induced by hypertonic sucrose was also similar in both cell lines. However, transferrin uptake was not completely blocked by hypertonic sucrose treatment, which disrupts clathrin-coated pit mediated endocytosis and virtually eliminates ASOR uptake in rat hepatocytes (39, 40). Since transferrin uptake also occurs through the clathrin-coated pit pathway (41), the partial inhibitory effect of hyperosmolarity indicates the existence of an alternate nonclathrin-mediated pathway for endocytosis of transferrin in these SK-Hep-1-derived cells. Nevertheless, these data show that the general endocytic pathways were the same in cell lines expressing either wild-type or palmitoylation-defective ASGP-Rs; they were not altered during the cell selection and cloning process.

Uptake of Surface-bound 125I-ASOR—We also determined the initial rates of internalization of one synchronized round of surface-bound ASOR and the sensitivity of the uptake to hyperosmolarity. The binding at 4°C of ASOR to ASGP-R in transfected SK-Hep-1 cells (not shown) or in hepatocytes (39) was not affected by 0.4 M sucrose. Cells expressing wild-type ASGP-Rs were able to internalize increasing amounts of ASOR with time after the cells were warmed to 37°C (Fig. 6). Roughly 60% of the surface-bound ligand was internalized at 12 min. In addition, the majority of this ASOR uptake was inhibited by hypertonic sucrose treatment, showing that most of the initial ASOR uptake mediated by wild-type ASGP-Rs occurred via the clathrin-coated pit pathway. In contrast, internalization of pre-bound ASOR was not detected in cells expressing nonpalmitoylated ASGP-Rs, showing that there was a defect in the rapid endocytosis of surface-bound ASOR. These results show that

Palmitoylation-defective ASGP-Rs was ~50% compared with cells expressing wild-type ASGP-Rs (Fig. 4C). The above results indicate that although cells expressing nonpalmitoylated ASGP-R are able to endocytose ASOR, they are defective in their ability to degrade the internalized ligands. In addition, cells expressing partially palmitoylation-defective ASGP-Rs, in
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Palmitoylation plays a role in regulating the rapid internalization (<12 min) of wild-type ASGP-Rs through the clathrin-coated pit pathway in transfected SK-Hep-1 cells.

Effect of Hyperosmolarity on Continuous Uptake of 125I-ASOR—The results in Fig. 5 suggested that there is also a nonclathrin-mediated pathway for the endocytosis of transferrin into SK-Hep-1 cells. This prompted us to examine the effect of hyperosmolarity on continuous ASOR uptake in the two stable cell lines. Cells expressing wild-type ASGP-Rs were sensitive to hyperosmolarity and internalized ~40% less ASOR in the presence of 0.4 M sucrose at 3 h compared with the control (Fig. 7A). In contrast, cells expressing palmitoylation-defective ASGP-Rs were resistant to sucrose treatment, since only a small percent, if any, of ASOR uptake was inhibited (Fig. 7B). These results indicate that in cells expressing palmitoylation-defective ASGP-Rs, almost none of the ASOR uptake is through clathrin-coated pits. The data also suggest that in SK-Hep-1 cells a nonclathrin-mediated pathway is utilized for the entry of both wild-type and nonpalmitoylated ASGP-R/ASOR complexes.

Effect of Hyperosmolarity on Degradation of 125I-ASOR—We next examined the effect of hyperosmolarity on the degradation of internalized ASOR by the SK-Hep-1 stable cell lines. Cells expressing wild-type ASGP-Rs were able to degrade ASOR, and this degradation was inhibited by the presence of hypertonic medium (Fig. 8A), even though the cells continued to endocytose ASOR (see Fig. 7A). Cells expressing nonpalmitoylated ASGP-Rs were barely able to degrade the internalized ASOR either in the presence or absence of sucrose (Fig. 8B). These results suggest that the hyperosmolarity-sensitive pathway is responsible for the ASOR degradation seen in cells expressing wild-type ASGP-Rs.

Co-localization of Internalized ASOR with Lysosomes—In order to assess whether the ligand degradation deficiency observed in cells expressing palmitoylation-defective ASGP-Rs was due to the failure to deliver ligand to lysosomes, we determined the subcellular localization of the internalized ASOR (Fig. 9). Under isotonic conditions, co-localization of internalized fl-ASOR with lysosomes was apparent in both cell lines (Fig. 9, A and C), although less co-localization was observed in cells expressing nonpalmitoylated ASGP-Rs. However, in cells treated with hypertonic sucrose, the internalized fl-ASOR was generally not present in lysosomes in either cell line (Fig. 9, E and G). These apparent differences were more evident when we...
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FIG. 7. Effect of hyperosmolarity on continuous 125I-ASOR uptake. Cells expressing wild-type (A) or nonpalmitoylated (B) ASGP-Rs were incubated with 1.5 μg/ml 125I-ASOR at 37 °C, in the presence (○) or absence (△) of 0.4 M sucrose, or a 100-fold excess of unlabeled ASOR (□). At the indicated times, cells were processed as described in the legend to Fig. 4 to determine the amount of ASOR uptake. Data are the average ± S.E. of three separate experiments.

FIG. 8. Effect of hyperosmolarity on degradation of 125I-ASOR. Cells expressing wild-type (A) or nonpalmitoylated (B) ASGP-Rs were incubated with 1.5 μg/ml 125I-ASOR at 37 °C, in the presence (○) or absence (△) of 0.4 M sucrose, or a 100-fold excess of unlabeled ASOR (□). At the indicated times, cells were processed as described in the legend to Fig. 4 to determine the amounts of degraded 125I-ASOR. Data are the average ± S.E. of three separate experiments.

quantified the extent of co-localization of the green (fl-ASOR) with red (lysosomes) pixels from similar confocal images. Under isotonic conditions, 73 ± 10% and 26 ± 10% of vesicles containing internalized fl-ASOR co-localized with lysosomes, respectively, in cells expressing wild-type or nonpalmitoylated ASGP-Rs (means ± standard deviations of 15 cells analyzed from different fields; p < 0.001, Student’s t test). In contrast, under hypertonic conditions, almost no co-localization of fl-

FIG. 9. Co-localization of internalized ASOR with lysosomes. Cell lines expressing wild-type (A, B, E, F) or palmitoylation-defective (C, D, G, H) ASGP-Rs were incubated with fl-ASOR and Lysotracker Red DND-99 in the presence (E, H) or absence (D, G) of 0.4 M sucrose as described under “Experimental Procedures.” Confocal fluorescence microscopy images (A, C, E, G) show staining of vesicles containing green fl-ASOR and the red lysosomal marker; the corresponding phase contrast images are in panels B, D, F, H. The white bar represents 20 μm.

ASOR with lysosomes was detected (<2%) in either cell line. These results show that the cells expressing nonpalmitoylated ASGP-Rs were very inefficient in the delivery of ligand to lysosomes, compared with cells expressing the wild-type ASGP-Rs. In addition, fl-ASOR internalized by either type of ASGP-R through the nonclathrin-mediated pathway was not delivered to lysosomes.

Rate of Dissociation of Internalized 125I-ASOR Complexes—Cells were pulse-labeled with 125I-ASOR and incubated with unlabeled ASOR for various times. After removing surface-bound ASOR, the total intracellular ASOR content (both free and ASGP-R-bound) and the fraction of ASOR that was still bound to receptors were then determined, respectively, by treating cells in the absence or presence of digitonin. More than 90% of the internalized ASOR was dissociated from the wild-type ASGP-Rs after 12 min, whereas only ~60% was dissociated from nonpalmitoylated ASGP-Rs (Fig. 10). The results indicate that the in vivo dissociation rate of ASOR-ASGP-R complexes was faster for wild-type ASGP-Rs compared with palmitoylation-defective ASGP-Rs.

Exocytosis of Internalized 125I-ASOR—Although ASOR was internalized under hypertonic conditions in transfected SK-Hep-1 cells expressing either wild-type or nonpalmitoylated ASGP-Rs, it was not delivered to lysosomes, and no degradation products were detected in the medium. Therefore, we examined the possible presence of a transcytosis pathway, by which ligands are internalized and transported across normally polarized cells without being degraded. The release of intact internalized ASOR into the medium was monitored in transfected SK-Hep-1 cells that had been pulse-labeled with 125I-ASOR. Under hypertonic conditions, cells expressing either wild-type or nonpalmitoylated ASGP-Rs released the same amounts (~40–50%) of internalized ASOR, as intact molecules, into the medium within 1 h (Fig. 11B). Comparatively less intact ASOR was externalized (<15%) from either cell line under isotonic conditions (Fig. 11A), but over the first 30 min the rate of intact ASOR release was 2-fold greater from cells expressing nonpalmitoylated ASGP-Rs compared with wild-type ASGP-Rs. These results show that a significantly higher percentage of ASOR, internalized via the clathrin-independent pathway, is subsequently externalized. Importantly, a higher level of ASOR externalization occurred with nonpalmitoylated ASGP-Rs, supporting the conclusion that their ligand processing and trafficking abilities are abnormal.

DISCUSSION

We have previously reported that both human ASGP-R subunits, H1 and H2, are post-translationally modified with pal-
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In a series of previous studies (20–24), we demonstrated that the State 2 ASGP-Rs are reversibly inactivated by treating cells with a variety of drugs that can also inactivate other recycling, endocytic receptors in various cell types (reviewed in Refs. 16, 17, 19). We also discovered that all ASGP-R subunits are palmitoylated (29, 30, 42) and that removal of the fatty acids from affinity-purified rat ASGP-Rs caused inactivation (loss of ligand binding activity) of the State 2, but not State 1, receptor population (26). Furthermore, State 2 ASGP-R inactivation in permeable hepatocytes (46) could be reversed by subsequent treatment with specific fatty acyl Co-A derivatives, which resulted in the reactivation of receptors (23, 24). Based on these and other similar results (20, 47), we proposed that during receptor-mediated endocytosis through the coated pit pathway, State 2 ASGP-Rs, as well as other recycling receptors, are transiently inactivated and then later reactivated before they recycle back to the cell surface (i.e. they undergo an I/R cycle). We suggested that this I/R cycle may be necessary for the efficient segregation and different intracellular routing of dissociated ligand and receptors, because it would eliminate the ability of ligand, at the predicted high concentration within endosomes, to rebind to receptors. Finally, we hypothesized that this I/R cycle was coupled to the fatty acylation status of State 2 ASGP-Rs. Modification of the cytoplasmic domains of the multimeric ASGP-Rs might regulate their activity, because the intercalation of fatty acyl chains into the membrane could alter the arrangement, and therefore ligand binding ability, of the extracellular carbohydrate binding domains (42).

Clearly, our present results do not support the latter hypothesis, because fatty acylation is not necessary for the ligand binding activity of the ASGP-R. The lack of palmitoylation also did not affect the intracellular distribution or routing of dissociated ligand and receptors, because it would eliminate the efficient segregation and different intracellular routing of dissociated ligand and receptors. Instead, we observed a slower rate of ASOR internalization by wild-type ASGP-R to a level similar to that of the nonpalmitoylated ASGP-R, which itself appears to have a slower rate of decay. This suggests that receptor palmitoylation does not affect the rate at which oligomeric complexes or individual subunits recycle to and from the cell surface.

Despite the extensive similarities between wild-type and nonpalmitoylated ASGP-Rs mentioned above, SK-Hep-1 cells expressing nonpalmitoylated ASGP-Rs displayed a slower rate of continuous ASOR uptake, dissociation and degradation, compared with cells expressing wild-type ASGP-Rs. We also demonstrated that SK-Hep-1 cells expressing nonpalmitoylated ASGP-Rs could internalize ASOR not only through the clathrin-dependent pathway, but also via an alternate, clathrin-independent route. ASOR internalization through noncoated pit pathways was not mediated by nonspecific adsorptive or fluid-phase endocytosis, but rather was specifically mediated by the ASGP-R, since SK-Hep-1 cells transfected with the plasmids alone did not accumulate ASOR. Hypertonic sucrose treatment reduced the rate of ASOR internalization by wild-type ASGP-R to a level similar to that of the nonpalmitoylated ASGP-R, which itself was resistant to such treatment.

Our results show that the greater ASOR uptake capacity in cells expressing wild-type, compared with palmitoylation-defective, ASGP-Rs was due to receptor-mediated uptake through clathrin-coated pits, and in turn suggest that palmitoylation of ASGP-Rs regulates their endocytosis and cellular routing via the clathrin-mediated pathway. Rates of ASOR uptake, dissociation, and degradation by cells expressing partially palmitoylation-defective ASGP-Rs, in which only one of the two ASGP-R subunits is not palmitoylated, were very similar to cells expressing ASGP-Rs with both subunits nonpalmitoylated. The residual ligand uptake and processing by all three types of palmitoylation-defective ASGP-Rs were equally resistant to

FIG. 10. Dissociation of internalized 125I-ASOR. Cells expressing wild-type (●) or nonpalmitoylated (▲) ASGP-Rs were pulse-labeled with 125I-ASOR, washed to remove surface-bound ligand, and then incubated with Hanks in the presence or absence of digitonin to measure, respectively, the amount of receptor-bound ligand and the total amount of cell-associated ligand as described under “Experimental Procedures.” Results were calculated as the percentage of total intracellular ASOR that was free (dissociated) and are shown as the average ± S.E. of three independent experiments, each performed in duplicate. The 7 and 12 min differences were both significant (p < 0.002, Student’s t test).

FIG. 11. Externalization of internalized 125I-ASOR. Cells expressing wild-type (●) or nonpalmitoylated (▲) ASGP-Rs were pulse-labeled with 125I-ASOR in the absence (A) or presence (B) of 0.4 M sucrose, and the externalization of intact 125I-ASOR was measured as described under “Experimental Procedures.” Results shown are the average ± S.E. of three independent experiments, each performed in duplicate. The 15 and 30 min differences in A were significant at a level of p < 0.00001 (Student’s t test).

mitic acid (29, 30, 42). In order to elucidate the role of palmitoylation in ASGP-R function, we constructed stable cell lines expressing either the wild-type, or mutant ASGP-Rs incapable of palmitate attachment to either one or both receptor subunits. The partial or complete lack of ASGP-R palmitoylation did not affect the steady-state distribution of cell surface and intracellular receptors among all the stable cell lines tested. Approximately 75% of the total cellular receptors were localized intracellularly, which is similar to the distribution of native ASGP-Rs in isolated rat hepatocytes (33, 43) and in HepG2 cells (44, 45). The wild-type and palmitoylation-defective ASGP-Rs also have comparable ligand-binding affinities, as well as similar profiles in vitro of dissociation of bound ligand as a function of pH.

In a series of previous studies (20–24), we demonstrated that the wild-type and palmitoylation-defective ASGP-Rs also have comparable ligand-binding affinities, as well as similar profiles in vitro of dissociation of bound ligand as a function of pH.
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hyperosmolarity. Therefore, palmitoylation of both the H1 and H2 subunits in hetero-oligomeric complexes appears to be necessary for the normal function of ASGP-Rs.

Nonpalmitoylated ASGP-Rs were almost completely defective in their ability to endocytose ligands via clathrin-coated pits under normal isotonic conditions, although a small percentage of ASOR molecules was still internalized and co-localized with lysosomes. However, only the cells expressing wild-type, but not nonpalmitoylated ASGP-Rs, were able to degrade the internalized ligand efficiently. This defect could be attributed to the low efficiency of ligand delivery to lysosomes in cells expressing nonpalmitoylated ASGP-Rs, as evidenced by the higher percentage of internalized ligands that are still bound to receptors (Fig. 10) and the lower percentage of internalized ASOR present in lysosomes (Fig. 9). Thus it appears that palmitoylation is required for the proper dissociation and sorting of internalized ASGP-R:ASOR complexes, which in turn allows the delivery of ligands to lysosomes, while the receptors are recycled to the cell surface. However, the pH-mediated dissociation of nonpalmitoylated ASGP-R:ASOR complexes in vitro was normal. Therefore, the dissociation defect of nonpalmitoylated ASGP-Rs could be due to impairment either of receptor delivery to the acidic compartment in which dissociation occurs or of the ability of receptor to trigger the normal acidification process within this compartment in vivo. This latter possibility seems unlikely and inconsistent with the co-localization of the ASGP-R with other recycling receptors in endosomal compartments (48). Alternatively, ligand dissociation in vivo may require something other than, or in addition to, a low pH environment, such as the proposed ASGP-R I/R cycle (16, 17, 19). Whatever its nature, the defect is related to the ASGP-R and not due to a clonal effect of the cell line isolated, because, as noted above, multiple cell lines expressing all three types of palmitoylation-defective ASGP-Rs exhibit very similar ASOR processing characteristics.3

Although the process of receptor-mediated endocytosis via the clathrin-coated pit pathway has been studied extensively for more than 30 years (49), mounting evidence from many different laboratories indicates that there are also alternative nonclathrin-coated endocytic pathways (50–52). For example, using the method of K+-depletion to inhibit clathrin-coated pit formation, Moya et al. (53) showed that endocytosis of diptheria toxin, but not ricin toxin, was inhibited in HEP-2 cells. Oka et al. (39) demonstrated that by inhibiting clathrin-coated pit formation in rat hepatocytes with hypertonic media, the uptake of ASOR was inhibited, whereas the fluid-phase uptake of Lucifer yellow was not affected. Moreover, Sandvig et al. (54) found that in various cell lines the endocytosis of transferrin and endothelial growth factor, but not the fluid-phase markers Lucifer yellow and ricin, were inhibited by lowering the cytosolic pH, which prevents clathrin-coated pits from pinching off from the plasma membrane. Thus, ample data show that clathrin-dependent and -independent pathways co-exist for the endocytosis of different types of molecules in various primary cells and established cell lines.

The hepatoma cell line SK-Hep-1 was selected for the stable expression of ASGP-Rs in the present study because it was originally derived from a patient with adenocarcinoma of the liver, and the cells show no endogenous expression of ASGP-Rs (37). Therefore, SK-Hep-1 cells could provide a cellular environment similar to that of hepatocytes and suitable for studying ASGP-R endocytosis and function. However, unlike the situation isolated hepatocytes or established hepatoma cell lines, in which >90% of ASGP-R internalization is inhibited by hypertonic medium and thus mediated by clathrin-coated pits (39), the transfected SK-Hep-1 cells endocytosed roughly equal numbers of wild-type ASGP-Rs via clathrin-dependent and -independent pathways. This unexpected result could be explained by the possibility that the hepatoma-derived SK-Hep-1 cell line may actually originate from an endothelial cell lineage. Characteristics of endothelial cells, such as abundant intermediate filaments, numerous endocytic vesicles, and electron dense granules consistent with Weibel-Palade bodies, have been detected in SK-Hep-1 cells (55). Plasmalemma invaginations at the plasma membrane constitute ~15–25% of the volume of many types of endothelial cells. These organelles do not possess a clathrin coat, and were later identified to be caveolae, which are implicated in the transcytosis of macromolecules across endothelial cells (50, 56).

Upon hypertonic sucrose treatment, ligands internalized via the clathrin-independent pathway were not delivered to lysosomes in cells expressing either wild-type or nonpalmitoylated ASGP-Rs (Fig 9). This is in contrast to other studies, which showed that ligands internalized through clathrin-dependent (e.g. α2-macroglobulin and transferrin) and clathrin-independent (e.g. cholera toxin and Con A) pathways were ultimately delivered to the same lysosomal compartments (50, 57, 58). Thus, the clathrin-independent pathway responsible for ASOR uptake in transfected SK-Hep-1 cells could be a transcytosis pathway. This possibility is supported by the results from the 125I-ASOR pulse-chase experiments in cell lines expressing wild-type or nonpalmitoylated ASGP-Rs (Fig 11). Under hypertonic conditions, ~50% of internalized ligands reappeared as intact molecules in the cell medium within 1 h, compared with only ~10% under isotonic conditions. Therefore, it is likely that the endocytosis of ASGP-Rs via the clathrin-independent pathway is mediated by a transcytosis pathway that has been implicated in the transport of macromolecules across endothelial cells (50, 56), from which the SK-Hep-1 cell line could have originated.

In summary, despite the complication of an unexpected clathrin-independent pathway in SK-Hep-1 cells, palmitoylation of the human ASGP-R plays an important role in regulating its endocytosis, dissociation and trafficking through the clathrin-coated pit pathway. Palmitoylation of the ASGP-R is necessary for the proper segregation of internalized ASOR and receptors, which leads to efficient delivery of ligands to lysosomes for degradation.

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