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The Human Asialoglycoprotein Receptor Is Palmitoylated and Fatty Deacylation Causes Inactivation of State 2 Receptors

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We report here for the first time that ASGP-Rs expressed in the human hepatoma cell lines HuH-7 and HepG2 are fatty acylated. Cells were metabolically labeled with [^{3}H]palmitate and active ASGP-Rs, which contain two subunits (HHL1 and HHL2), were purified by affinity chromatography and subjected to nonreducing SDS-PAGE and fluorography. [^{3}H]Palmitate was covalently incorporated into both HHL1 and HHL2. When gel slices containing HHL1/HHL2 were treated at neutral pH with 1 M hydroxylamine, but not 1 M Tris, >95% of the radioactivity was removed, indicating that the attachment of palmitate to ASGP-Rs is to cysteines. Furthermore, the same mild hydroxylamine treatment caused partial ASGP-R inactivation; 50–70% of receptors corresponding to the previously characterized State 2 ASGP-Rs were inactivated. We conclude that both HHL1 and HHL2 are covalently modified by fatty acylation, which may regulate the ligand-binding activity of human State 2 ASGP-Rs. We propose that fatty acylation/deacylation of cytoplasmic domains is a general mechanism by which extracellular ligand-binding activity of oligomeric transmembrane receptors can be regulated.

The mammalian ASGP-R is an integral transmembrane glycoprotein that binds galactose-or N-acetylgalactosamine-terminal glycoconjugates (1,2). Appropriate ligands, such as ASOR, are bound by ASGP-Rs, endocytosed and intracellularly processed; after dissociation and segregation the ligand is delivered to lysosomes for degradation and the receptor is then recycled back to the cell surface. In isolated rat hepatocytes State 2 ASGP-Rs, one of two receptor subpopulations, undergoes a novel inactivation/reactivation cycle as they traverse the intracellular receptor recycling pathway (2,3). In permeable rat hepatocytes or human hepatoma cells, the activity of this same State 2 ASGP-R population is reversibly regulated in the absence of cytosol by ATP and fatty acyl-CoAs (4,5). These observations led us to suggest that this ASGP-R inactivation/reactivation cycle could regulate receptor activity during endocytosis and receptor recycling. In recent studies, we demonstrated that the three subunits of rat ASGP-Rs are covalently modified by fatty acylation (6) and this modification is directly involved in the regulation of receptor ligand-binding activity (7).

The functional human ASGP-R is a hetero-oligomeric complex composed of two subunits, HHL1 and HHL2, with molecular masses of 45 and 50 kDa, respectively (8). Since the amino acid sequences of HHL1 and HHL2 are highly homologous to those of RHL1 and RHL2/3 (9), we examined whether the human ASGP-R is also modified by fatty acylation and whether this modification is involved in regulating receptor activity. Here we show that both subunits of the human ASGP-R are palmitoylated via thioester linkages to Cys. Furthermore, mild hydroxylamine treatment deacylates the receptor and inactivates State 2 ASGP-Rs.

METHODS

Materials. Human orosomucoid, CNBr-activated Sepharose 4B, neuraminidase (Type X), Triton X-100, akaline phosphatase conjugated to anti-rabbit IgG, and standard fatty acids were from Sigma. ^{125}I-ASOR was prepared by desialylation

Abbreviations: ASGP-R, asialoglycoprotein receptor; ASOR, asialo-orosomucoid; HHL1, HHL2, human hepatic lectin subunits 1 and 2; CRD, carbohydrate recognition domain; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; RHL, rat hepatic lectin; Tris, tris(hydroxymethyl)aminomethane.

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of orosomucoid with neuraminidase and subsequent iodination (10). $Na^{125}I$ (10-20 mCi/μg of iodine) was from Amersham Corp. 1,3,4,6-tetrachloro-3α,6α-diphenylglycouril (IODO-GEN) was from Piece Chemical Company. Hydroxylamine was from Aldrich. [9,10(n)^{3}H]Palmitic acid (56 Ci/mmol) was from New England Nuclear. Fluoro-HANCE was from Research Products Int. Nitrocellulose (0.1 μm and 0.45 μm) was from Schleicher & Schuell. 5-Bromo-4-chloro-3-indolyl phosphate, nitroblue tetrazolium, and all chemicals for electrophoresis were from Bio-Rad. All other chemicals were reagent grade.

Cell culture. Cell lines were cultured by reported procedures (11,12) with some modifications. The human hepatoma cell line HuH-7 was cultured in MEM (GIBCO, catalog number 41500-034), containing 10% fetal calf serum, 2 mM glutamine, 50 mU/ml insulin, 100 mU/ml penicillin, and 100 μg/ml streptomycin. The human hepatoma cell line HepG2 was cultured in 50% Dulbecco's modified Eagle's medium (GIBCO, catalog number 12100-046) and 50% Ham's F12 (GIBCO, catalog number 21700-075) containing 10% fetal calf serum, 2 mM glutamine, 50 mU/ml insulin, 100 mU/ml penicillin, and 100 μg/ml streptomycin. Both cell lines were cultured at 37°C in a 5% CO_2 atmosphere.

Metabolic labeling of cells. At ~90% confluency, cell monolayers (60 mm dishes) were rinsed once with 2 ml serum-free medium, and preincubated in this medium at 37°C for 1 h. The radio-labeling was then carried out with 400 μCi/ml [9,10(n)^{3}H] palmitic acid at 37°C for 4–16 h in medium supplemented with 10% FCS that had been extensively dialysed against PBS (6).

Affinity purification and electrophoresis. Unlabeled or [^{3}H]palmitate-labeled cells were scraped off the dishes and collected in a conical tube. The cells were permeablized with 0.2% digitonin, washed, solubilized in 1.5% Triton X-100, and active ASGP-Rs were purified by affinity chromatography using ASOR-Sepharose (7). ASGP-Rs were eluted either with an equal volume of 2× Laemmli sample buffer (13) at room temperature for 20 min or with an EGTA-containing elution buffer (7). Samples were analyzed by SDS-PAGE using 12% (w/v) gels.

Hydroxylamine treatment. After SDS-PAGE, the receptor-containing gel lanes were incubated at 25°C with 1 M Tris (pH 7.4) or 1 M hydroxylamine (pH 7.4) for 2 h, and the gels were then dried and visualized by fluorography as described (6). To determine the effect of hydroxylamine on receptor activity, EGTA-eluted ASGP-Rs were purified from unlabeled cells, incubated with 1 M Tris (pH 7.4) or 0–1 M hydroxylamine (pH 7.4) on ice for 4 h, and the samples were analyzed by SDS-PAGE/Western blotting or by ligand-binding as described below.

Ligand-binding assay. The ligand-binding activity of untreated or hydroxylamine-treated ASGP-Rs was determined by a dot-blot assay using ^{125}I-ASOR (7). Nonspecific binding was determined by assessing the bound radioactivity remaining in the presence of a 75-fold excess of nonlabeled ASOR. All binding assays were done in duplicate.

General. Western blotting was performed (14), using a specific polyclonal antibody (IgG fraction) raised against the SDS-denatured nonreduced CRD of RHL1.[1] SDS-PAGE was carried out by the method of Laemmli (13). Fluorography was performed as described previously (6). ^{125}I-Radioactivity was measured using a Packard 5002 Cobra 2γ spectrometer.

RESULTS AND DISCUSSION

To examine whether human ASGP-Rs are covalently modified by attachment of long chain saturated fatty acids, both hepatoma cell lines were metabolically labeled with [^{3}H]palmitate, and active ASGP-Rs were purified and analysed by SDS-PAGE under nonreducing conditions. [^{3}H]Palmitate was incorporated into both HHL1 and HHL2, from both HepG2 (Fig. 1) and HuH-7 (Fig. 2) cells. Identification of [^{3}H]palmitate-labeled bands as HHL1 and HHL2 was confirmed by rehydration of the exposed gel lane and subsequent Western blotting analysis with a CRD-specific antibody (Fig. 1, lane 5; Fig. 2, lane 4). This experiment also showed no incorporation of palmitate into other copurified proteins.

Fatty acids may be attached to proteins through N-linked amide bonds, O-linked ester bonds or S-linked thioester bonds (15). Ester and thioester linkages, but not amide linkages, are labile to akaline treatment (15,16). Thioester linkages are particularly sensitive to hydroxylamine. When gels containing HHL1 or HHL2 isolated from HepG2 or HuH-7 cells were treated with 1 M Tris or 1 M hydroxylamine, >95% of the ^{3}H-radioactivity was removed (Figs. 1 and 2, lanes 3). Control treatment with 1 M Tris did not significantly reduce the radioactivity in either subunit (Figs. 1 and 2; lanes 2). This result indicates that palmitate is attached to Cys residues of HHL1 and HHL2 as a thioester.

During metabolic labeling, palmitate could be converted into other fatty acids or metabolites. To assess this possibility, [^{3}H]palmitate-labeled, affinity-purified ASGP-Rs were separated by SDS-PAGE, and subjected to akaline treatment. Over 90% of the radioactivity was recovered in the

[1] F-Y Zeng and P.H. Weigel, manuscript in preparation.

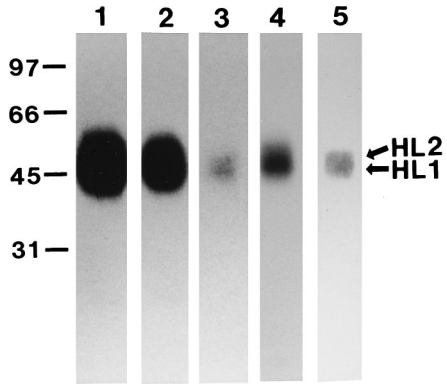

FIG. 1. Metabolic labeling of active ASGP-Rs in HepG2 cells with [^{3}H]palmitate. Human hepatoma HepG2 cells (three 60 mm dishes) were radiolabeled for 16 h, and active ASGP-Rs were purified and analyzed by SDS-PAGE on a 12% gel under nonreducing conditions. Gel slices (lanes) were treated with 10 mM Hepes, pH 7.4, 150 mM NaCl, 10 mM EGTA alone (lane 1) or with 1 M Tris (lane 2) or 1 M hydroxylamine (lane 3) at 22°C for 2 h prior to fluorography. The gel was exposed to X-ray film at −70°C for 25 days (lanes 1–3). The sample in lane 4 was the same as lane 1, except the exposure time was shorter (10 days). After exposure, the gels from lane 1 were rehydrated and analyzed by Western blotting with the anti-CRD antiserum (lane 5). Although not apparent in this blot, the broad HHL band is sometimes resolved into two bands.

organic phase, indicating that almost all fatty acids are attached to the protein through ester bonds. Analysis of the akaline-released fatty acids by reverse-phase HPLC (6) showed that >80% of the radioactivity was recovered as palmitic acid (not shown). No significant radioactivity was detected in the positions of stearic acid or myristic acid.

We previously discovered and characterized the existence of two distinct ASGP-R populations that mediate ligand uptake and processing by two different intracellular pathways (2). These two receptor populations have been designated State 1 and State 2 ASGP-Rs. The number of State 2 and State 1 ASGP-Rs are roughly equal, but about 80% of the cellular endocytic work load (ligand uptake and degradation) is mediated by the State 2 receptors. The State 2 ASGP-R population undergoes transient inactivation/reactivation during receptor recycling and the activity and/or cellular distribution of these receptors is modulated by a variety of drugs or treatments in intact cells. State 1 ASGP-Rs are not affected. These two ASGP-R populations and pathways described in rat hepatocytes are also found in human hepatoma cells. Although others have not emphasized these conclusions, the data obtained with rat hepatocytes and human hepatoma cells are essentially identical (2,8).

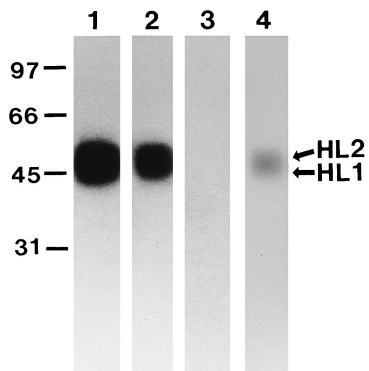

FIG. 2. Metabolic labeling of active ASGP-Rs in HuH7 cells with [^{3}H]palmitate. HuH7 cells were grown, labeled and ASGP-Rs were purified and analyzed as described in Methods and the legend to Fig. 1. Lane 1, no treatment; lane 2, Tris-treated; lane 3, hydroxylamine-treated; lane 4, Western blot of lane 1.

Most significantly, Stockert *et al.* (17) isolated a mutant HuH-7 cell line that is defective in the State 2 ASGP-R pathway, but still has a functional State 1 ASGP-R pathway. We previously suggested (2,3) that the State 1/State 2 pathways are a common feature for multiple endocytic receptors, not just ASGP-Rs. This Trf1 mutant (trafficking mutant 1), in fact, has a pleiotropic defect that affects multiple receptors and provides genetic evidence for the existence of the two receptor subpopulations we have characterized.

We have demonstrated the direct involvement of fatty acylation in the regulation of ligand-binding activity of the rat ASGP-R (7). Mild hydroxylamine treatment almost completely releases covalently linked palmitate from rat ASGP-Rs and concurrently inactivates only the State 2 receptors (6,7). Thus, it was important to verify that human ASGP-Rs have the same differential sensitivity to hydroxylamine as rat ASGP-Rs, since this differential sensitivity reflects differences in the State 1/State 2 ASGP-R populations. Using a dot-blot assay, we examined the effect of hydroxylamine on the ^{125}I-ASOR-binding activity of ASGP-Rs freshly purified from human HuH-7 and HepG2 cells. Under mild conditions (pH 7.4, 4°C, 4h) hydroxylamine treatment caused ASGP-R inactivation in a dose-dependent but clearly biphasic manner (Fig. 3). About 40% of ASGP-Rs were inactivated with 0.3 M hydroxylamine. Increasing concentrations in the range of 0.3–1.0 M resulted in only a slightly greater inactivation. About 35% of the total ASGP-Rs were still active even after exposure to 1 M hydroxylamine. Control treatment with 1 M Tris did not decrease ASGP-R activity (Fig. 3).

To determine whether the loss of ASGP-R activity by hydroxylamine treatment corresponds to depalmitoylation of receptor, freshly purified [^{3}H]palmitate-labeled ASGP-Rs from HEpG2 or HuH-7 cells were treated with 1 M hydroxylamine at 4°C for 2 h prior to SDS-PAGE. This treatment, which results in ~60% ASGP-R inactivation (Fig. 3), indeed released >90% of the metabolically incorporated [^{3}H]palmitate (not shown: the results were identical to the experiments in Figs. 1 and 2; lanes 3). By comparison, Tris treatment released only <10% of the radiolabel (lanes 2). These results show that both HHL1 and HHL2 are depalmitoylated by hydroxylamine and that ASGP-R inactivation is concomitant with receptor depalmitoylation. Under these conditions, hydroxylamine did not cause polypeptide cleave as measured by reducing SDS-PAGE (not shown). Based on the present study, we suggest that the biological function of both human and rat ASGP-Rs *in vivo* is regulated by a reversible acylation/deacylation cycle.

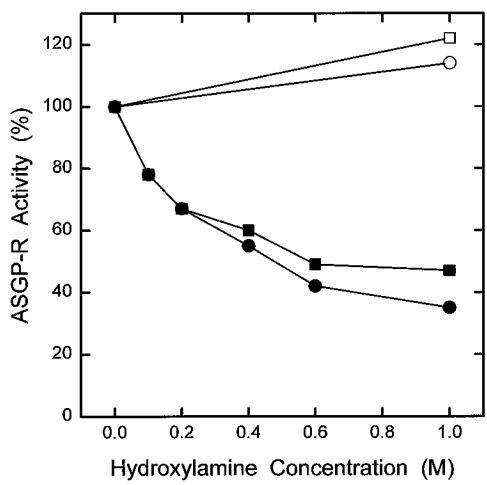

FIG. 3. Hydroxylamine treatment causes ASGP-R inactivation. Freshly purified ASGP-Rs from HuH-7 (○, ●) or HepG2 (□, ■) cells were incubated with 10 mM Hepes, pH 7.4, 150 mM NaCl, 20 mM $CaCl_2$ in the presence of 1 M Tris (○, □) or 0 to 1 hydroxylamine (■, ●) on ice for 4 h. The ^{125}I-ASOR-binding activity was then determined by a dot blot assay.

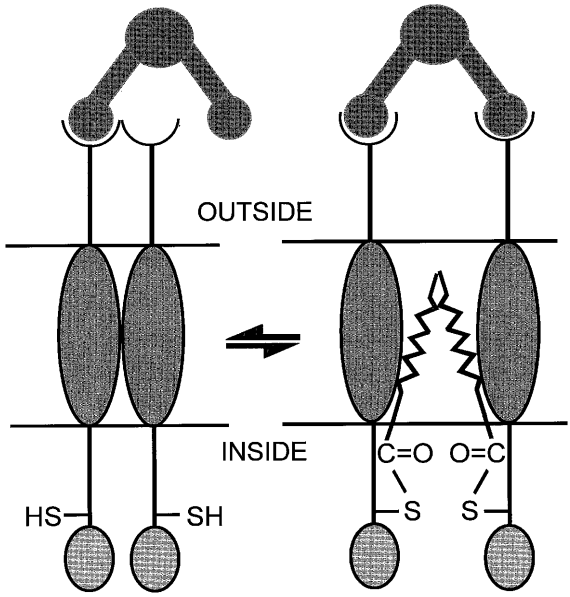

FIG. 4. Regulation of multimeric receptor activity by fatty acid acylation. The model depicts a molecular basis for how fatty acid acylation on the cytoplasmic domain of a receptor can alter the extracellular ligand-binding domains. Intercalation of the fatty acid acyl chains among the interacting transmembrane protein domains would change the relative spacing and geometry of the external ligand-binding domains, resulting in altered ligand recognition. The figure shows the active state as being fatty acylated.

All three subunits of the rat ASGP-R are covalently modified by fatty acylation (6). Here we demonstrate that both human ASGP-R subunits are also palmitoylated. Palmitate is incorporated into HHL1 and HHL2 to similar extents through hydroxylamine-labile thioester linkages, presumably to a Cys residue in each subunit. The cytoplasmic domains of HHL1 and HHL2 contain one and two Cys, respectively, close to the transmembrane domain, which are the likely sites for palmitoylation. Both subunits also have a Cys in the transmembrane domain that could be fatty acylated.

Fatty acid acylation of the cytoplasmic domains of oligomeric receptors could be a general mechanism by which cells achieve the transmembrane regulation of extracellular ligand-binding activity (Fig. 4). Most, if not all, cell surface receptors are oligomeric and contain multiple transmembrane subunits. Usually, as with the ASGP-R, high affinity ligand binding requires the participation of extracellular domains from more than one transmembrane subunit arranged in a precise spatial organization. Fatty acylation near the cytoplasmic domain/membrane junction would lead to insertion of the fatty acyl chains into the membrane and intercalation among the transmembrane-spanning domains of a receptor. Intercalated fatty acyl chains would alter the spatial arrangement of subunits within the membrane and consequently also the arrangement of the external ligand-binding domains. Different oligomeric receptors could be either active or inactive in the fatty acylated state. The key feature of this model is that changing the acylation state of cytoplasmic domains in a multimeric transmembrane receptor will change the spatial arrangement of extracellular domains constituting the ligand-binding site and thereby alter the receptor's ability to bind its ligand.

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