Fatty Acylation of the Rat Asialoglycoprotein Receptor

THE THREE SUBUNITS FROM ACTIVE RECEPTORS CONTAIN COVALENTLY BOUND PALMITATE AND STEARATE*

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Rat hepatic asialoglycoprotein receptors (ASGP-Rs) are hetero-oligomers composed of three homologous glycoprotein subunits, designated rat hepatic lectins (RHL) 1, 2, and 3. ASGP-Rs mediate the endocytosis and degradation of circulating glycoconjugates containing terminal N-acetylgalactosamine or galactose, including desialylated plasma glycoproteins. We have shown in permeable rat hepatocytes that the ligand binding activity of one subpopulation of receptors (designated State 2 ASGP-Rs) can be decreased or increased, respectively, by ATP and palmitoyl-CoA (Weigel, P. H., and Oka, J. A. (1993) J. Biol. Chem. 268, 27186–27190). We proposed that a reversible and cyclic acylation/deacylation process may regulate ASGP-R activity during endocytosis, receptor-ligand dissociation, and receptor recycling. In the accompanying paper (Zeng, F.-Y., and Weigel, P. H. (1995) J. Biol. Chem. 270, 21382–21387), we show that the ligand binding activity of affinity-purified State 2 ASGP-Rs is decreased by treatment with hydroxylamine under mild conditions consistent with these ASGP-Rs being fatty acylated in vivo. In this study, we used a chemical method to determine the presence of covalently-bound fatty acids in individual ASGP-R subunits. The affinity-purified ASGP-R preparations were separated by SDS-polyacrylamide gel electrophoresis under nonreducing conditions, and the gel slices containing individual RHL subunits were treated with alkali to release covalently bound fatty acids, which were subsequently analyzed by gas chromatography and confirmed by gas chromatography-mass spectrometry. Both stearic and palmitic acids were detected in all three receptor subunits. Pretreatment of ASGP-Rs with hydroxylamine before SDS-polyacrylamide gel electrophoresis reduced the content of both fatty acids by 66–80%, indicating that most of these fatty acids are attached to cysteine residues via thioester linkages. Furthermore, when freshly isolated hepatocytes were cultured in the presence of [3H]palmitate, all three RHL subunits in affinity-purified ASGP-Rs were metabolically labeled. We conclude that RHL1, RHL2, and RHL3 are modified by fatty acylation in intact cells.

Covalent binding of long chain saturated fatty acids occurs with a wide variety of membrane proteins post-translationally and significantly influences protein localization and/or function (1). Two of the most common modifications involve acylation with myristate and palmitate. Myristate is usually attached to an N-terminal glycine via an amide bond in a relatively stable linkage. In contrast, palmitate is attached to thiol group of cysteines via a thioester bond or to serine residues via an ester bond; both linkages are reversed by alkali treatment (2). Palmitoylation of many proteins has been shown to be a dynamic process (3, 4).

The hepatic asialoglycoprotein receptor (ASGP-R) mediates the endocytosis of desialylated glycoproteins containing terminal galactose or N-acetylgalactosamine residues (5, 6). The functional rat ASGP-R is a hetero-oligomer composed of three subunits, RHL1, RHL2, and RHL3, with molecular masses of 41,500, 49,000, and 54,000 Da, respectively (7). The amino acid sequences of all three subunits are closely related, and they are the products of two different genes (8). RHL1 is the major subunit of the ASGP-R, while RHL2 and RHL3, encoded by the same gene, are minor subunits and differ only in the type and amount of post-translational carbohydrate modification (9).

We and others have previously demonstrated that the ASGP-R ligands are endocytosed and intracellularly processed by two functionally different receptor populations via two distinct pathways (6, 10). We have designated these two receptor populations as the State 1 ASGP-Rs and the State 2 ASGP-Rs. In intact cells, the State 2 ASGP-Rs undergo a transient inactivation/reactivation cycle during receptor recycling (11). This cycle has been successfully reconstituted in permeable rat hepatocytes (12–14). In permeabilized cells the State 2 ASGP-Rs are inactivated by the addition of ATP in a time- and temperature-dependent manner (12); these ATP-inactivated receptors can then be quantitatively reactivated by the addition of palmitoyl-CoA (13, 14).

In the accompanying paper (15), we also demonstrate that the activity of one population of affinity-purified ASGP-Rs, the State 2 receptors, is selectively inactivated by treatment with hydroxylamine, a chemical frequently used to release thioester-linked fatty acids from proteins. These above results suggest that one or all ASGP-R subunits are modified by fatty acylation in vivo and that a reversible acylation/deacylation process may be involved in regulating the ligand-binding activity of ASGP-Rs as they function during receptor mediated endocytosis.

In this study, we have used gas chromatography-mass spectrometry to examine directly whether total (State 1 plus State 2) ASGP-Rs contain covalently-bound fatty acids. The results...
demonstrate that all three ASGP-R subunits contain covalently-linked palmitate and stearate. Treatment of each RHL subunit with hydroxylamine under mild conditions released both fatty acids in relatively large amounts, indicating that most fatty acids are attached via thioester linkages.

**EXPERIMENTAL PROCEDURES**

Materials—Human orosomucoid, CNBr-activated Sepharose 4B, Tri-ton X-100, neumaminidase (Type X), phenylmethylsulfonyl fluoride, leupeptin, pepstatin A, soybean trypsin inhibitor, the standard fatty acids for HPLC analysis, and water (HPLC grade) were from Sigma. Boron trifluoride (BF₃)-methanol was from Fluka, and concentrated hydrochloric acid was prepared by the treatment of anhydrous hydrochloric acid with neumaminidase (16). Ammonium persulfate, SDS, Tris, glycine, N,N-dimethylbenzylami- lydine, and SDS-PAGE molecular weight markers were from Bio- Rad. BSA and protein assay reagents were from Pierce. HPLC-grade methanol, hexane, and chloroform were from Mallinkrodt. Acrylamide (twice recrystallized) was from U. S. Biochemical Corp. Hydroxylamine was from Aldrich. The standard methyl esters of fatty acids were from Matreya, Inc., Pleasant Gap, PA. [9,10-³H]Palmitic acid (56 Ci/mmol) was from DuPont NEN. Fluoro-HANCE was from Research Products Int. All other chemicals were high purity reagent grade.

Preparation of Active ASGP-Rs—Isolated hepatocytes from male Sprague-Dawley rats (Harlan Breeding Laboratories, Houston, TX, and Sasco) were prepared by the treatment of orosomucoid with neuraminidase as described in the accompanying paper (15). The esters were separated on a DB23 column (30 m long, 0.2 mm film thickness, under split mode) and flame ionization detector (21). The temperature of the injector port and detector was set at 200 and 300 °C, respectively. Ultra high purity grade nitrogen, used as carrier gas at a flow rate 0.5 ml/min, was passed through molecular sieve (4 Å) and silica gel in order to remove the water and moisture, respectively. The fatty acid methyl esters were analyzed using a thermal gradient from an initial column temperature of 150 °C to a final temperature of 225 °C with an increase of 10 °C/min. The peak area of the fatty acid methyl esters was determined using the Hewlett-Packard 3396A integrator and the fatty acid methyl esters were quantitated by comparison to the response of known standards of fatty acid methyl esters.

Gas Chromatographic-Mass Spectrometric Analysis of Fatty Acid Methyl Esters—To confirm the identity of the fatty acids, fatty acid methyl esters were also analyzed by GC-MS using a Varian 3400 gas chromatograph interfaced with a Fennigkin Inoce-50 mass spectrometer at the Analytical Chemistry Center, University of Texas Health Science Center, Houston, TX. The esters were separated on a DB 23 column (30 m long, 0.2 mm film thickness, under split mode) and flame ionization detector (21). The temperature of the injector port and detector was set at 200 and 300 °C respectively. Ultra high purity grade nitrogen, used as carrier gas at a flow rate 0.5 ml/min, was passed through molecular sieve (4 Å) and silica gel in order to remove the water and moisture, respectively. The fatty acid methyl esters were analyzed using a thermal gradient from an initial column temperature of 150 °C to a final temperature of 225 °C with an increase of 10 °C/min. The peak area of the fatty acid methyl esters was determined using the Hewlett-Packard 3396A integrator and the fatty acid methyl esters were quantitated by comparison to the response of known standards of fatty acid methyl esters.

**Analytical Briefings of Radioactive Fatty Acid Metabolism**

Gas chromatography—No evidence of significant metabolism of fatty acids was observed in male-, female-, or neonatal Sprague-Dawley rats. Male Sprague-Dawley rats were administered either naturally occurring or radiolabeled fatty acids and were sacrificed 2–4 h later. The body fat was excised, weighed, and pooled. Lipids were extracted using chloroform/methanol (1:2, v/v) and the lipids were analyzed by mass spectrometry. Radioactivity was determined by liquid scintillation counting.

**Other Experiments**

**Human Ovarian Carcinoma**

To determine if hydroxylamine treatment releases covalently-bound fatty acids, the purified ASGP-R preparation was incubated with 1 n hydroxylamine or 1 n Tris (as a control) at pH 7.4 on ice for 2 h before the addition of sample buffer and SDS-PAGE.

**Release of Covalently-Bound Fatty Acids by Chemical Treatments—** All glassware was new and thoroughly rinsed with chloroform/metha- nol (1:1, v/v) prior to use. HPLC-grade water was used throughout the procedure. Affinity-purified ASGP-R (≤ 400 nCi of [³H]palmitate—Freshly iso- lated rat hepatocytes were plated onto 60-mm dishes (~ 2 × 10⁶ cells/dish) and cultured at 37 °C with 5% CO₂ in William's Medium E as described previously (17). The medium was supplemented with 10% fetal calf serum, 10 mg/ml Heps, pH 7.4, 2 mg/ml L-glutamine, 50 mg/ml insulin, 50 mg/ml penicillin, 100 μg/ml streptomycin, and 50 μg/ml dexamethasone. For labeling experiments, cells were preincubated with medium without fetal calf serum at 37 °C for 2 h. The radioactive fatty acid methyl esters were added to the culture medium at a final concentration of 200 μl of [³H]palmitate and 10% fetal calf serum (dialyzed against phosphate-buffered saline). The active ASGP-Rs from radiolabeled cells were purified by affinity chromatography using asialo-orosomucoid-Sepharose as described in Ref. 15, and separated by SDS-PAGE as described above. The analysis of radiolabeled fatty acids was carried out as described above. The analysis of [³H]labeled fatty acids was carried out as described above. The analysis of radioactivity was determined by liquid scintillation counting. The retention times of the radioactive peaks were compared with those of the standard fatty acids.

Gas chromatography—The protein content was measured by the method of Bradford (22) using BSA as standard. Western blotting was carried out as described elsewhere, using subunit-specific antibodies (23). For fluorography, the fixed gel was washed with water for 30 min, and then soaked.
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These background peaks were present even in gels on which no samples had been loaded and could not be eliminated by changing the experimental conditions. The intensities of these peaks were also significantly increased by increasing the gel size analyzed (data not shown). For this reason, we used the same size blank or subunit-containing gels (verifed by weight) to minimize the effect of differing gel size on the results. To verify that SDS-PAGE and the washing procedures after SDS-PAGE could effectively remove noncovalently bound fatty acids, BSA which is known to contain many kinds of noncovalently bound fatty acids, was subjected to the same procedure. The result showed no detectable fatty acids in either the chloroform/methanol wash or the alkaline hydrolyzate in comparison with a blank gel (not shown), indicating that SDS-PAGE and the subsequent wash procedures (before alkali treatment) completely removed noncovalently bound fatty acids.

The analysis of alkaline hydrolysates of purified ASGP-R by GC-MS clearly demonstrated the presence of palmitate and stearate in each of the three RHL subunits (Figs. 1 and 2). One GC peak in these samples was identified as the methyl ester of palmitate by two criteria: First, its retention time (11.65 min) on gas chromatography was identical to that of standard methyl palmitate and this peak co-chromatographed with the standard methyl palmitate. Second, mass spectrometric analysis of this GC peak (retention time 11.65 min) gave a molecular ion (m/z = 270), which is the expected mass of methyl palmitate. Furthermore, additional ion mass peaks at 74 (McLafferty ion), 87, and 143, are the expected major molecular fragment ions from the methyl esters of saturated fatty acids. The mass spectrum of the 11.65-min peak was identical to that of the standard methyl palmitate (Fig. 2A). The methyl ester of stearate was identified by the same criteria. All samples contained a GC peak with a retention time of 13.86 min, identical to that of standard methyl stearate, and GC-MS analysis revealed a molecular ion (m/z = 298) and other major ions characteristic (21, 24) of the methyl ester of stearate (Fig. 2B). In addition to methyl palmitate and methyl stearate, small amounts of the methyl esters of myristic, oleic, and linoleic acids were also detected by GC in the alkaline hydrolysates of all three subunits (Fig. 1). Palmitate and stearate methyl esters were not detected in the chloroform/methanol wash of the gels containing ASGP-R subunits (Fig. 1, left panel). These esters were only found after processing the alkaline hydrolysates. After alkali treatment, the incubation of the gels containing receptor subunits with strong acid should release amide-linked fatty acids. The analysis of the acid hydrolysates of RHL1 and RHL2 by GC showed no detectable methyl esters of any fatty acid above background (data not shown), indicating the absence of amide-linked fatty acids in these two receptor subunits. RHL3, however, gave significant amounts of palmitate and stearate (≈6 times background).

To determine if palmitate and stearate are attached to Cys residues of the receptor subunits via thioester linkages, the affinity-purified ASGP-Rs were treated prior to SDS-PAGE with hydroxylamine under mild conditions. Total ASGP-R was first treated with NH₂OH, and then each RHL subunit was purified by SDS-PAGE and treated with alkali (Table I). Quantification of the released fatty acids showed that hydroxylamine treatment released 73, 100, and 68% of the methyl palmitate that could be detected in RHL1, RHL2, and RHL3, respectively. Similarly, for methyl stearate 74, 100, and 79% of this fatty acid detectable in RHL1, RHL2 and RHL3, respectively, was released by NH₂OH treatment. Tris treatment had no significant effect on any of the RHL subunits (Table I). These results indicate that most (>70%) of the covalently bound fatty acids in an alkali-labile linkage are attached to each RHL subunit via
FIG. 2. Mass spectrometric analysis of the GC peaks identified as palmitate and stearate methyl esters. The peaks at 11.65 min (A) and 13.85 min (B) from the GC analysis of fatty acids released from individual RHL1, RHL2, and RHL3 subunits as shown in Fig. 1 were derivatized and analyzed by GC-MS. The mass spectra of both standard fatty acid methyl esters are also shown. The major ion peaks (such as m/z = 74 (M+Cl eff) and 87, and 143) that are characteristic of fatty acid methyl esters were found in both GC peaks from all RHL samples. Ion peaks unique to methyl palmitate (m/z = 227, 239, and 270 (M+)) or to methyl stearate (m/z = 255, 267, and 298 (M+)) were found in each sample as indicated.
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**Table I**

| Methyl palmitate | Methyl stearate |
|------------------|-----------------|
| RHL1 | nmol | RHL2 | nmol | RHL3 | nmol | RHL1 | nmol | RHL2 | nmol | RHL3 | nmol |
| Experiment 1 | 8.5 | 9.3 | 10.5 | 17.6 | 16.1 | 19.6 |
| Experiment 2 | 9.5 | 9.8 | 8.5 | 13.0 | 16.4 | 15.4 |
| Experiment 3 | 17.2 | 8.1 | 12.5 | 13.4 | 9.9 | 10.4 |
| 1 M Tris | 15.6 (91)* | 7.8 (96) | 11.8 (94) | 11.4 (85) | 9.1 (92) | 9.9 (95) |
| 1 M NH₂OH | 4.7 (27) | 0.0 (0) | 4.0 (32) | 4.4 (26) | 0.0 (0) | 2.7 (21) |

*Values in parentheses are percentages recovered relative to the control values of experiment 3.

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Freshly purified ASGP-Rs were subjected to SDS-PAGE, extraction, and alkaline hydrolysis as described under “Experimental Procedures.” The released fatty acids were derivatized to their methyl esters, and analyzed by GC. The values for methyl palmitate and methyl stearate were obtained by comparing the area of corresponding peaks with those of known amounts of the standard fatty acid methyl esters (after subtracting the background for a blank gel of the same size). Three experiments were performed using independent ASGP-R preparations. To determine if pretreatment with hydroxylamine released fatty acids, equal amounts of ASGP-R from the same preparation (experiment 3) were incubated on ice for 2 h with buffer alone (10 mM Hepes, pH 7.4, 150 mM NaCl, 6.7 mM KCl, 0.025% Triton X-100), or buffer containing 1 mM Tris or 1 mM hydroxylamine. These treated samples were subsequently subjected to the same procedures as above to determine the remaining alkaline-releasable fatty acid content.

| Methyl palmitate | Methyl stearate |
|------------------|-----------------|
| RHL1 | nmol | RHL2 | nmol | RHL3 | nmol |
| Experiment 1 | 8.5 | 9.3 | 10.5 | 17.6 | 16.1 | 19.6 |
| Experiment 2 | 9.5 | 9.8 | 8.5 | 13.0 | 16.4 | 15.4 |
| Experiment 3 | 17.2 | 8.1 | 12.5 | 13.4 | 9.9 | 10.4 |
| 1 M Tris | 15.6 (91)* | 7.8 (96) | 11.8 (94) | 11.4 (85) | 9.1 (92) | 9.9 (95) |
| 1 M NH₂OH | 4.7 (27) | 0.0 (0) | 4.0 (32) | 4.4 (26) | 0.0 (0) | 2.7 (21) |

*Values in parentheses are percentages recovered relative to the control values of experiment 3.

DISCUSSION

A number of diverse proteins including viral proteins (25), the α subunits of G proteins (26), the G protein-coupled receptors (27–29), and the transferrin receptor (30, 31) have been shown to be modified by palmitate via thioester linkages. Palmitoylation has been suggested to play an important functional role in many proteins. For example, the palmitoylation of subunits of G proteins is involved both in membrane attachment as well as in regulating signaling capability (32, 33). Palmitoylation of the neuronal growth cone protein GAP-43 reduces its ability to catalyze nucleotide exchange on a G protein (34). Many investigators have shown that palmitoylation/depalmitoylation of a variety of proteins is a dynamic process (3, 4).

Although the present study shows for the first time that ASGP-Rs are fatty acylated, Stockert found evidence over 10 years ago that [¹³C]palmitate might be incorporated into the human ASGP-R. Our results here clearly demonstrate that all three subunits of the ASGP-R are modified by fatty acylation. The GC-MS analysis shows the presence of both palmitate and...
The ASGP-R are metabolically labeled by [3H]palmitic acid. In initial metabolic labeling studies with [3H]stearate in each RHL subunit. The results from the GC-MS analyses were confirmed by the finding that all three subunits of the ASGP-R are metabolically labeled by [3H]palmitic acid. Initial metabolic labeling studies with [3H]stearate did not show incorporation into ASGP-Rs, although [3H]palmitate was readily incorporated. In earlier studies, however, stearyl-CoA was as effective as palmitoyl-CoA in being able to reactivate State 2 ASGP-Rs that had been inactivated by ATP treatment of permeable cells (13). Furthermore, as shown in Table I and the accompanying paper (15), the sensitivity of fatty acid release by hydroxylamine treatment at neutral pH is indicative of a thioester linkage. Pretreatment with hydroxylamine released approximately 66–100% of the stearate or palmitate in RHL1, RHL2, and RHL3 that could be released by alkaline hydrolysis. Therefore, approximately 66% of the covalently bound fatty acids in alkali-labile linkages is probably present as thioesters. This percentage could be as high as 80%. The finding that one population of purified ASGP-Rs is selectively inactivated by hydroxylamine treatment, under mild conditions that releases essentially all of the covalently associated fatty acids, (15), supports this hypothesis. Thus, we conclude that the three ASGP-R subunits are modified by palmitate and stearate, and that acylation-deacylation of the ASGP-R could directly regulate its activity.

An important point about the fatty acylation results presented here is that we analyzed the total active ASGP-R pool, which contains both State 1 and State 2 receptors. It is likely that the State 1 ASGP-Rs are not fatty acylated; fatty acylation may occur exclusively in the State 2 ASGP-R population (15). Further studies will address whether fatty acylation in RHL1, RHL2, and/or RHL3 is the molecular basis for the two functionally distinct receptor populations and how fatty acylation/deacylation regulates receptor activity.

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