Fatty Acylation of the Rat and Human Asialoglycoprotein Receptors

A CONSERVED CYTOSOLMIC CYSTEINE RESIDUE IS ACYLATED IN ALL RECEPTOR SUBUNITS*

(Received for publication, August 16, 1996, and in revised form, September 23, 1996)

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Functional rat or human asialoglycoprotein receptors (ASGP-Rs) are hetero-oligomeric integral membrane glycoproteins. Rat ASGP-R contains three subunits, designated rat hepatic lectins (RHL) 1, 2, and 3; human ASGP-R contains two subunits, HHIL1 and HHIL2. Both receptors are covalently modified by fatty acylation (Zeng, F.-Y., Kaphalia, B. S., Ansari, G. A. S., and Weigel, P. H. (1995) J. Biol. Chem. 270, 21382–21387; Zeng, F.-Y., Oka, J. A., and Weigel, P. H. (1996) Biochem. Biophys. Res. Commun. 218, 325–330). We report here that the single Cys residue in the cytoplasmic domain of each RHL or HHIL subunit is fatty acylated. The degree of acylation is 90% per subunit. Deacylation of affinity-purified ASGP-Rs with hydroxylamine results in the spontaneous formation of dimers through reversible disulfide bonds, indicating that deacylation concomitantly generates free thiol groups. Reaction of hydroxylamine-treated ASGP-R with [14C]iodoacetamide resulted in the specific incorporation of radioactivity into all RHL and HHIL subunits, verifying that fatty acids are attached via thioester linkages. To identify the Cys residue involved in the thioester linkages, 14C-carboxymethylamidemethylated RHL subunits were separated by SDS-polyacrylamide gel electrophoresis and digested in-gel with trypsin, and the resulting peptides were separated by reverse-phase high performance liquid chromatography. Amino acid sequence of radioactive peptides revealed that Cys35 in RHL1 and Cys47* in RHL2 and HHIL3 were radiolabeled and, therefore, are fatty acylation sites. Fatty acylation of HHIL subunits was analyzed by site-directed mutagenesis. Metabolic labeling of Cos7 cells transfected with wild type HHIL1 cDNA resulted in substantial incorporation of [3H]palmitate into purified HHIL1. Incorporation of [3H]palmitate into a C368 mutant of HHIL1 was negligible (~1%) compared with wild type. This result also shows that Cys57 within the transmembrane domain of HHIL1 is not normally palmitoylated. We conclude that Cys35 in RHL1, Cys47 in RHL2 and HHIL3, and Cys57 in HHIL1 are fatty acylated. Cys57 in HHIL1 and probably Cys56 in HHIL2 are not palmitoylated.

The hepatic asialoglycoprotein receptor (ASGP-R)1 has been a good model system for studying receptor-mediated endocyto-

* This research was supported by National Institutes of Health Grant GM 49695. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: ASGP-R, asialoglycoprotein receptor (hASGP-R or rASGP-R denote the human or rat ASGP-R); ASOR, asialo-orosomucoid; RHL1, RHL2, RHL3, rat hepatic lectin 1, 2, or 3; HHIL1, HHIL2, human hepatic lectin 1 or 2; PAGE, polyacrylamide gel electrophoresis; CRD, carbohydrate recognition domain; HPLC, high performance liquid chromatography.
within the transmembrane domain. In addition HHL2 contains a third nonconserved Cys at position 58.

Cys residues at or near the junction between cytoplasmic and transmembrane domains are frequently potential palmitoylation sites. Such cysteines are palmitoylated in many membrane proteins such as the transferrin receptor (17), the G protein-coupled receptors (18, 19), the HLA-D-associated invariant chain (20) and the influenza virus hemagglutinin (21).

We recently found that all subunits in both rat and human ASGP-Rs are fatty acylated (13, 14). In both cases [3H]palmitate could be readily incorporated into active ASGP-Rs, and mild treatment with hydroxylamine caused fatty acid deacetylation and concomitant loss of lipid binding activity. Although the chemical sensitivity of thioester linkages to cleavage by hydroxylamine is an indication that this labile bond is present, it is not proof. Normal ester bonds, for example involving Ser or Thr, or even peptide bonds, can be cleaved by hydroxylamine if these bonds are under appropriate conformational strain. In order to understand the molecular mechanism by which the fatty acylation of ASGP-R is regulated by fatty acids, we have identified the fatty acylation sites of the three receptor subunits. Using a novel chemical method combined with site-directed mutagenesis, our results show that the single Cys in the cytoplasmic domain of each subunit is palmitylated, whereas the conserved transmembrane domain Cys is not.

**EXPERIMENTAL PROCEDURES**

**Materials**—Hydroxylamine was from Aldrich. Human orosomucoid, iodoacetamide, CNBr-activated Sepharose 4B, acetonitrile (HPLC grade), trifluoroacetic acid, and all proteinase inhibitors were from Sigma. [14C]Iodoacetamide (60 mCi/mmol) was from Amersham Corp. [9,10-3H]Palmitic acid (56 Ci/mmol) was from DuPont NEN. ASOR was prepared by treatment of orosomucoid with neuraminidase (22). Trypsin and chymotrypsin (protein sequence grade) were from Boehringer, Mannheim. Subtilisin (catalogue number, 572908) was from Calbioch. Nitrocellulose membrane (0.1 μm) was from Schleicher & Schuell. Polyvinylidene difluoride membrane (0.2 μm) and all chemicals for electrophoresis were from Bio-Rad. Fluoro-hance was from Research Products International Corp. All other chemicals were reagent grade.

**Preparation of Active ASGP-Rs**—Isolated hepatocytes from Sprague-Dawley rats (Sasco, Oklahoma City, OK) were prepared by a modification (23) of a collagenase perfusion procedure (24). The cells were first incubated in medium 1/bovine serum albumin at 37°C for 3 h, and expressed HHL1 protein was purified by affinity chromatography using ASOR-Sepharose 4B as described previously (12).

**Site-directed Mutagenesis**—Mutants were generated using Promega’s Altered Site®II in vitro mutagenesis system according to the manufacturer’s instructions. Plasmids pGA1, containing full-length HHL1, and pKA2b, containing full-length HHL2b (29), were generously provided by Dr. Martin Spiess (University of Basel, Switzerland). HHL1 cDNA was subcloned into pALTER Ex-1 Vector (Promega) using HindIII and EcoRI. HHL2 cDNA was subcloned into pALTER Ex-1 Vector using BamHI and EcoRI. The Cys residues in the cytoplasmic and transmembrane domains of HHL1 were replaced by Ser residues, yielding the single mutants HHL1(C36S) and HHL1(C57S). The mutagenic (sense) oligonucleotides used were the following (the altered nucleotides are in bold): 5'-CTG CAG CGT TTC GAC TGG CTA GGA CCT CGG C-3' (C36S); and 5'-CTG GGT GTC AGG ATG TCA GTC GTA C-3' (C57S). All mutations were verified by DNA sequencing.

**Transfection and Metabolic Labeling**—Wild type and mutant HHL1 cDNAs were subcloned into the pcDNA3.1 (+) expression vector using HindIII and EcoRI restriction sites. For transfection, plasmid DNA was purified from Escherichia coli Top10F using the EndoFree plasmid maxi kit. COS-7 cells (obtained from ATCC) were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal calf serum, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin in an atmosphere of 5% CO2 at 37°C. Cells were transfected at ~50% confluence with pcDNA3.1 (+) plasmids using a calcium phosphate transfection kit according to the manufacturer’s instructions. Cells were labeled 24 h posttransfection with 400 Ci/ml [3H]palmitic acid from active rat or human ASGP-Rs, suggesting that this palmitate is attached to cysteine residues through thioester linkages (12, 14). To investigate whether the linkage of fatty acids to individual subunits, hydroxylamine-treated rat ASGP-Rs were analyzed by SDS-PAGE under nonreducing conditions.
probably a contaminant.

...weight band in (25) sample buffer (125 mM Tris-HCl, pH 6.8, 20% glycerol, 2% SDS) possible chemical degradation of receptor subunits (12). Samples were a t 3° C for 1 h (lane 2), 2 (lane 4), or 4 h (lane 6), or at room temperature for 1 h (lane 6) or at 37° C for 1 h (lane 7). EGTA was included in the reaction to prevent possible chemical degradation of receptor subunits (12). Samples were removed, mixed with an equal volume of 2-fold concentrated Laemmli (25) sample buffer (125 mM Tris-HCl, pH 6.8, 20% glycerol, 2% SDS) (B) or without (A) 5% (v/v) β-mercaptoethanol, boiled for 2 min, and subjected to SDS-PAGE and silver staining. Dimers are absent from the area in B marked by a line from A. The minor high molecular weight band in all lanes of B migrates slower than the dimers and is probably a contaminant.

As shown in Fig. IA, hydroxylamine treatment of rASGP-Rs at pH 7.4 results in the formation of several new protein bands, primarily in the range of 90–110 kDa, in a time-dependent manner. The control treatment with Tris had no effect. A small amount of larger oligomers was also observed. Reduction with β-mercaptoethanol completely abolished these larger bands (Fig. 1B) and regenerated the normal RHL subunits, indicating that these new, larger proteins were formed via disulfide bonds after hydroxylamine treatment. Taking the molecular masses into account, most of the newly formed proteins are likely to be dimeric RHLs. Disulfide bonds could form between any two RHL subunits if free thiol groups are generated after removal of fatty acids. The same behavior is found with purified hASGP-Rs, which also form dimers upon hydroxylamine treatment (Fig. 2).

To determine the composition of the dimeric RHLs, we performed Western analyses using two receptor subunit-specific antibodies (33), raised against synthetic peptides corresponding to the C-terminal 17 amino acids of RHL1 and RHL2/3 (Fig. 3). The 90 kDa-band was recognized by the RHL1-specific antibody but not by the RHL2/3-specific antibody, whereas the broad band at 95–110 kDa reacted with the RHL2/3-specific antibody but not the RHL1-specific antibody (Fig. 3). These results clearly show that deacylation of active rASGP-Rs with hydroxylamine under mild conditions results in the formation of predominantly homodimers (RHL1-RHL1 and RHL2/3-RHL2/3). A similar analysis of the disulfide-bonded dimer composition from hASGP-R (Fig. 2) was not feasible, since the HHL1 and HHL2 subunits are not usually resolved by SDS-PAGE.

We interpret the above results to mean that spontaneous disulfide bond formation occurs after fatty deacylation of ASGP-R subunits. The assumption that the dimers formed during hydroxylamine treatment were deacylated was confirmed by examining ASGP-Rs that had been metabolically labeled with [3H]palmitate (Fig. 2 and Fig. 4). After hydroxylamine treatment, essentially all of the palmitate was removed from the monomeric HHL or RHL subunits, and no radioactivity was found with the dimeric subunits (Fig. 2A, lane 2 and Fig. 4B, lane 2).
in active ASGP-R preparations (Fig. 5B, lane 1). Treatment with 1 mM hydroxylamine resulted in extensive incorporation of radioactivity into all three subunits (Fig. 4B, lane 3). The control incubation in the presence of 1 mM Tris, pH 7.4, gave a much smaller increase in the amount of labeled RHL subunits (Fig. 5B, lane 2). Densitometric quantitation showed that the increase with Tris was about 19% of the hydroxylamine effect.

The effect of Tris treatment reflects the lability of thioesters and our previous findings that freshly purified State 2 ASGP-Rs lose activity with storage (12). Essentially identical results were obtained with purified hASGP-R (not shown). These results show that [14C]iodoacetamide specifically labels hydroxylamine-generated free thiol groups and no other functional groups in the ASGP-R. This finding became the basis of our biochemical approach to determine the sites of thioester-linked fatty acylation.

The stoichiometry of fatty acylation was estimated from these radiolabeling experiments based on the specific radioactivity of [14C]iodoacetamide and the amount of ASGP-R analyzed. RHL1, RHL2, and RHL3 were estimated to contain an average of 0.94, 1.0, and 1.3 fatty acyl groups/subunit, respectively. This stoichiometry of approximately one fatty acid per subunit is consistent with our previous gas-liquid chromatography-mass spectrometry results (13).

The single Cys residue in the cytoplasmic domain of RHL1, RHL2/3, and HHIL1 is a potential fatty acylation site. The fact that the N-terminal amino acids of the three RHL subunits are blocked precluded direct determination of the 14C-carboxyamidomethylated Cys by N-terminal sequence analysis of intact subunits. However, we have observed that partial digestion of ASOR-Sepharose complexes with subtilisin in the presence of Ca2+, KCl, 10 mM CaCl2, and 0.05% Triton X-100) at room temperature for 5 min. The reaction was terminated by three rapid washes with cold buffer B containing 0.1 mM phenylmethylsulfonyl fluoride. The bound proteins from the untreated (lane 1) or subtilisin-treated (lane 2) ASGP-R:ASOR-Sepharose were removed with EGTA elution buffer (12) and subjected directly to nonreducing SDS-PAGE followed by silver staining (lanes 1 and 2) or Western blotting with the RHL1-specific antibody (lane 3) or the RHL2/3-specific antibody (lane 4), or to treatment with [14C]iodoacetamide plus 1 mM hydroxylamine prior to SDS-PAGE and fluorography as described in Fig. 4 (lane 5).

The same approach failed to identify palmitoylation sites of the minor RHL2 and RHL3 subunits, since subtilisin readily digested both proteins into small peptides (Fig. 6). To determine their palmitoylation sites, 14C-carboxyamidomethylated RHL2/3 subunits (prepared as in Fig. 5) were subjected to in-gel digestion with trypsin. The recovered peptides were separated by reverse-phase HPLC, and radioactive fractions were identified (Fig. 7). RHL2 (Fig. 7B) and RHL3 (Fig. 7C) showed similar peptide maps, and radioactivity was detected in fractions 4 (peak 1) and 15/16 (peak 2). Repurification of the combined fractions 15 and 16 using a shallower gradient gave only a low recovery and no significant improvement in purity (not shown). RHL1 gave a different peptide map (Fig. 7A) with radioactivity in fractions 4 (peak 1), 27 (peak 2), and 29 (peak 3). The individual radioactive fractions were directly analyzed by N-terminal sequencing (Table 1). For RHL2 and RHL3, fractions 15 and 16 gave the same sequences, both consisting of two peptides in a molar ratio of 3:1 to 2.1. The major peptide was matched with Thr35-Glu-Asn-Pro-Arg38 (RHL2/3 cleaved at Arg34-Thr), and the minor peptide was Leu52-Cys-Ser-Lys56 which represents RHL2/3 cleaved at Arg52-Leu (Table 1). Radioactivity was detected only in the second cycle, indicating that this cycle contained 14C-carboxyamidomethylated cysteine. These results demonstrate that Cys52 of RHL2 and RHL3 is modified by attachment to fatty acid. Fraction 4 (P1) in all cases (Fig. 7, A–C) probably contains free [14C]iodoacetamide, since no peptide sequence could be detected.
The sequence analysis of peak 2 from RHL1 also gave two peptides in a molar ratio of about 3:1. The major peptide corresponds to Leu\(^{103}\)-Val-Glu-Ser-Gln-Leu-Glu-Lys\(^{110}\) of RHL1 (cleaved at Lys\(^{102}\)-Leu). The minor peptide was identified as Leu\(^{34}\)-(Cys)-Ser-Gly-Phe\(^{38}\) derived from RHL1 by cleavage at Arg\(^{35}\)-Leu (Table I). The second residue of this peptide should be Cys, and as expected, radioactivity was detected only in the second cycle. Peak 3 gave a major sequence corresponding to Gln\(^{75}\) through Lys\(^{89}\) of RHL1 and several minor peptides at similar molar ratios that have not been further analyzed, but radioactivity was detected only in the second cycle. These results are consistent with the above sequence analysis of the 40-kDa fragment (seen in Fig. 6) and verify that Cys\(^{35}\) in RHL1 is fatty acylated. Thus, the single cysteine residue in the cytoplasmic domains of all three rat subunits is identified as an essentially stoichiometric fatty acylation site. Similar attempts to isolate and sequence \(^{14}\)C-labeled peptides from hASGP-R, prepared as in Fig. 5, failed. The above studies did not determine whether the transmembrane Cys, which is conserved in all RHL and HHL subunits, is fatty acylated. No radiolabeled peptides of this region could be identified after hydroxylamine treatment and reaction with iodoacetamide. On the other hand, freshly purified ASGP-R from human or rat showed no significant reactivity with this reagent, suggesting that either no free —SH groups are present in active receptor or any free Cys residues are inaccessible to the alkylating agent. No reaction with iodoacetamide could be demonstrated even in the presence of SDS.

To resolve this uncertainty, we used site-directed mutagenesis to change Cys to Ser in the cytoplasmic and transmembrane domains of HHL1. This subunit is expressed well alone when COS-7 cells are transfected with a cDNA encoding wild type protein (Fig. 8A, lane 1). The expressed protein is readily detected after metabolic labeling with \(^{3}\)H)palmitate (Fig. 8B, lane 1). In contrast, the HHL1(C36S) mutant incorporates virtually no palmitate, although the protein is expressed as well as the wild type (Fig. 8, lanes A2 and B2). This result shows that Cys\(^{36}\) in HHL1 is fatty acylated, like the homologous Cys\(^{35}\) in RHL1. Additionally, dimer formation was much greater for wild type HHL1 compared with HHL1(C36S), probably reflecting greater spontaneous deacylation and disulfide bond formation during the purification of HHL1 containing Cys\(^{36}\).

Since mutation of the single cytoplasmic Cys completely abolishes palmitoylation of the protein, we also conclude, therefore, that transmembrane Cys\(^{57}\) is not normally palmitoylated. Interestingly, neither the single HHL1(C57S) mutation nor the double HHL1(C36S,C57S) mutant was stably expressed in COS-7 cells. Little or no HHL1 protein could be detected. Although transmembrane Cys\(^{57}\) is not palmitoylated and not involved in a disulfide bond, it may be necessary for assembly of HL subunits into a stable oligomeric ASGP-R.

DISCUSSION

The human or rat ASGP-Rs consist, respectively, of two or three polypeptide subunits that are the products of two different genes (15). RHL2 and RHL 3 have the same core polypeptide, differing only in the type and extent of carbohydrate modification. The primary structures of RHL1 and RHL2/3 show a high degree of identity. Each subunit has 10 cysteine residues at conserved positions (15), of which one is located in the N-terminal cytoplasmic domain, one in the transmembrane domain, and eight in the extracellular domain; the latter form four intra-chain disulfide bonds (35). In addition, HHL2 has a Cys at position 58 (1). Our results (Fig. 5) confirm the conclusion that all extracellular cysteines in RHL1 participate in the formation of disulfide bonds. Previous studies demonstrated that the rat ASGP-R is covalently modified by palmitic acid and

![Fig. 7](image-url)
fluorography (rified HHL1 was analyzed by nonreducing SDS-PAGE followed by [3H]palmitate from purified ASGP-Rs (not shown).

*Fig. 3. The C36S mutant of HHL1 is not palmitoylated. COS-7 cells were transfected with cDNAs encoding wild type HHL1 (lanes A1 and B1) or HHL1(C36S) (lanes A2 and B2) and metabolically labeled with [3H]palmitate as described under “Experimental Procedures.” Purified HHL1 was analyzed by nonreducing SDS-PAGE followed by fluorography (B) or Western blotting (A) with rabbit antiserum to rASGP-R. The arrow indicates the broad HHL band at 35–40 kDa.*

Fatty Acylation Sites of ASGP-Rs

The physiological significance of ASGP-R subunit dimer formation upon release of fatty acids was not addressed in this study and remains unknown. It raises the interesting possibility, however, that changes in the number of fatty acylated Cys residues (or the pattern of acylated subunits) within the oligomeric ASGP-R could lead to the formation or cleavage of disulfide bonds between subunits. Such changes could clearly alter the conformation of both internal and external domains of the receptor and lead to new or altered interactions with other regulatory proteins (e.g., protein kinases or thioesterases) or with ligand. Although several studies have demonstrated that the functional rat and human ASGP-Rs are hetero-oligomers (2, 38, 39), the structure of the native ASGP-R remains debated. Halberg et al. (40) showed only homo-oligomeric products in cross-linking experiments using hepatocyte microsomes, suggesting that no physical association of RHL1 and RHL2/3 exists. That we observe essentially exclusive formation of homodimeric RHL1-RHL1 or RHL2/3-RHL2/3 upon hydroxylamine treatment of affinity-purified ASGP-Rs suggests that rASGP-R exists in solution either in a homo-oligomeric form or in a particular hetero-oligomeric form in which like subunits are closer to, or more likely to react with, each other. Due to the lack of resolution between HHL1 and HHL2, similar data and conclusions for the human ASGP-R cannot be obtained.

As noted in Fig. 3, some oligomeric RHLs that are probably RHL1 trimers also formed upon hydroxylamine treatment of ASGP-Rs. These oligomers appear to be disulfide-bonded, since reducing agents convert them to monomeric RHLs. Freshly purified active ASGP-Rs do not contain interchain disulfide bonds, even though the transmembrane Cys residues are free. Formation of trimers after deacylation with hydroxylamine could be explained if the removal of fatty acid chains attached to the cytoplasmic domains enhanced the ability of two adjacent transmembrane cysteines to form a disulfide bond. One subunit could be disulfide-bonded to a second subunit through Cys−Cys linkages and to a third subunit through a Cys−Cys linkage.

We have proposed that a reorganization or altered packing of transmembrane domains occurs when oligomeric receptors are fatty acylated or deacylated in their cytoplasmic domains because the acyl chains will intercalate into the membrane among the transmembrane domains (14). Changes in the relative spacing of these membrane domains, as acyl chains are added or removed, would translate into changes in the spacing of the external CRDs and regulate ligand binding. The cytoplasmic Cys residues are palmitoylated in many proteins (34), and the transmembrane cysteines in some proteins (usually near the cytoplasmic domain junction), such as the transferrin receptor (41) and the cell surface glycoprotein CD4 (42), are also modified by fatty acids. Our mutagenesis results, however, show that the Cys residue in the transmembrane domain of HHL1 subunits is not palmitoylated.

In the present study we find that native ASGP-Rs contain close to one thioacyl group per subunit. Even though the transmembrane Cys is not palmitoylated, freshly purified ASGP-R is not carboxyamidomethylated despite partial denaturation of ASGP-Rs with SDS or by heating prior to alkylation. Either this Cys is not accessible to the polar reagent or the residual secondary and tertiary structure of the protein renders this thiol group nonreactive with iodoacetamide. Our biochemical and mutagenesis results show that Cys36 in RHL1, Cys54 in RHL2/3, and Cys50 in HHL1 are modified by fatty acids. Saxena and Fallon (43) have reported preliminary results that HHL2 is likely palmitoylated at Cys54 as well. It appears then...
that the same fatty acylated Cys residue is conserved in all subunits in both rat and human ASGP-Rs.

When the location of the acylation sites in the three RHL and two HHL subunits is compared with that in other integral membrane proteins in which Cys is near the transmembrane and cytoplasmic domain junction, it is clear that there is no unique or common requirement for the spacing of the Cys relative to the transmembrane domain. In the case of RHL1 and RHL2/3, the Cys is located four amino acids from the transmembrane domain; this distance is six amino acids in the vesicular stomatitis G protein (44), the influenza virus hemagglutinin (21), and the 63-kDa integral membrane protein (45). In contrast, there are one and two amino acid distances between the cytoplasmic Cys and the transmembrane domains identified in the cell surface glycoprotein CD4 (42) and the HLA-D-associated invariant chain (20), and 12–13 amino acid distances in the human β2-adrenergic receptor (18) and bovine rhodopsin (46). There are also no known consensus sequences for the palmitoylation of membrane proteins although the use of the HPLC, Dr. Ken Jackson for help with peptide sequencing, Anil Singh for preparing hepatocytes, and Debbie Blevins for help preparing the HPLC, Dr. Ken Jackson for help with peptide sequencing, Anil Singh for preparing hepatocytes, and Debbie Blevins for help preparing the manuscript.

Acknowledgments—We thank Bing Xia for technical assistance, Janet A. Oka for assistance with cell culture, Dr. Richard Cummings for use of the HPLC, Dr. Ken Jackson for help with peptide sequencing, Anil Singh for preparing hepatocytes, and Debbie Blevins for help preparing the manuscript.

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