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A Specific Antibody to the Carbohydrate Recognition Domain of the Asialoglycoprotein Receptor RHL1 Subunit Does Not React with RHL2/3 but Blocks Ligand Binding

Fu-Yue Zeng,[1] Janet A. Oka, and Paul H. Weigel[2]
Department of Biochemistry & Molecular Biology, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma 73190

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The rat asialoglycoprotein receptor (ASGPR) is believed to be a hetero-oligomer composed of three subunits, designated rat hepatic lectin 1, 2, and 3 (RHL1, 2, and 3). The carbohydrate recognition domains (CRDs) of RHL1 and RHL2/3 are 56% identical. We developed a polyclonal antibody that specifically recognizes the CRD of RHL1 but not RHL2/3. When purified ASGPRs were bound to ligand-Sepharose, the CRD of RHL1, but not RHL2 or RHL3, was resistant to digestion with subtilisin. Antibody against purified RHL1 CRD recognized only RHL1 in Western blot analysis of crude cell extracts or purified receptors without detectable cross-reaction to RHL2/3. Although it does not recognize the CRD of RHL2 or RHL3, this antibody specifically inhibited 80–90% of the cell surface or total cellular ^{125}I-ASOR binding to isolated rat hepatocytes and >90% of ligand binding to purified rat ASGPRs. The antibody also immunoprecipitates active ASGPRs containing all three RHL subunits. The results indicate that homo-oligomeric RHL2/3 complexes, able to bind ASOR, do not form on hepatocytes by subunit rearrangement.

The mammalian hepatic asialoglycoprotein receptor (ASGPR[3]) mediates the endocytosis and intracellular processing of glycoconjugates containing terminal galactosyl or N-acetylgalactosaminyl residues (1-3). The rat ASGPR consists of three polypeptide subunits, designated RHL1, RHL2 and RHL3. The amino acid sequences of these subunits are closely related, and they are the products of two different genes (4). RHL1 is the major subunit. RHL2 and RHL3 are minor subunits with identical core proteins that differ only in the type and extent of posttranslational carbohydrate modification (5). Each subunit contains four domains: an N-terminal cytoplasmic domain, a transmembrane domain, an extracellular stalk or neck, and a C-terminal carbohydrate recognition domain (CRD). A major difference between RHL1 and RHL2/3 is the presence of a unique 18 amino acid sequence in the N-terminal cytoplasmic domain of RHL2/3. The C-terminal CRDs of RHL1 and RHL2/3 (~134 amino acids) show a high extent of sequence identity (~56%).

There is presently conflicting information about the oligomeric organization of ASGPRs. Mice lacking the minor subunits MHL2/3, still stably express MHL1 on the surface of hepatocytes, although these homo-oligomeric ASGPRs do not mediate internalization and degradation of a ligand such as ASOR either in the animal or in isolated hepatocytes (6, 7). Cells transfected with cDNAs for RHL1 (8) or HHL1 (9) also express these homo-oligomers on their surfaces and can specifically bind ligand, although at a lower affinity (1-10%) than the hetero-oligomers, whose K_ds are 1-5 nM (1-3). For example, COS-7 cells transfected with cDNA for HHL1 express ~2.5 x 10^6 binding sites/cell for ASOR with a K_d = 40 nM (9). Internalization and degradation of ASOR bound by these homo-oligomeric receptors in transfected cells is very inefficient and essentially not detectable.

In contrast to the above results, crosslinking experiments and immunoprecipitation studies suggested that native rat (10, 11) and human (12) ASGPRs are hetero-oligomers. Both RHL1 and RHL2/3 subunits are required for the ability to bind and endocytose ligands such as ASOR (8). However, crosslinking studies using rat liver microsomes, detected only homo-oligomeric crosslinked products, such as RHL1-RHL1 (4). Thus, the subunit composition of functional ASGPRs still re-

[1] Present address: Laboratory of Bioorganic Chemistry, NIDDK/NIH, Bldg. 8A, Room, BIA-09, Bethesda, MD 20892.

[2] To whom correspondence should be addressed. E-mail: paul-weigel@OUHSC.edu.

[3] Abbreviations used: ASGPR, asialoglycoprotein receptor; ASOR, asialo-orosomucoid; RHL, rat hepatic lectin; HHL, human hepatic lectin; CRD, carbohydrate recognition domain.

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mains largely debated. Affinity purified rat ASGPR preparations contain the three subunits in approximate ratios of 3-4:1:1 (for RHL1:RHL2:RHL3). We previously suggested (3) that the native ASGPR is a hetero-hexamer composed of a core of four RHL1 subunits with two RHL2/3 subunits on opposite sides of the hexamer. This structure could potentially form two trimeric binding sites per ASGPR. Ruiz and Drickamer (13) have also suggested that RHL1 forms a ligand-binding core in the hetero-oligomeric receptor. Recently the 80 amino acid connection between the CRD and transmembrane domains of HHL1 was shown to form a trimer by itself or a hetero-tetramer in the presence of the analogous HHL2 domain (14). The HHL2 domain alone formed a tetramer. These results raise the possibility that rearrangement of subunits at the cell surface or in endosomal compartments could give rise to three types of ASGPR species; two homo-oligomers (of RHL1 or RHL2/3) and a hetero-oligomer.

To investigate further this question of ASGPR organization, we produced a novel antibody against the CRD of RHL1 that specifically recognizes only RHL1 without cross-reaction to RHL2/3. The results indicate that RHL1 and RHL2/3 have very different susceptibilities to subtilisin when hetero-oligomeric ASGPRs are bound to ligand and that the CRDs of RHL1 and RHL2/3 display different epitopes for immunologic recognition.

MATERIALS AND METHODS

Materials. Human orosomucoid, CNBr-activated Sepharose 4B, neuraminidase, β-mercaptoethanol, subtilisin A, trypsin, and alkaline phosphatase conjugated to goat anti-rabbit IgG were from Sigma. $Na^{125}I$ (10-20 mCi/μg of iodine) was from Amersham Corp. ^{125}I-ASOR was prepared by desialylation of orosomucoid with neuraminidase and subsequent iodination as described previously (15). Nitrocellulose was from Schleicher & Schuell. SDS, 5-bromo-4-chloro-3-indolyl phosphate, nitroblue tetrazolium, and all chemicals for electrophoresis were from Bio-Rad. All other chemicals were reagent grade.

Purification of active ASGPRs. Isolated hepatocytes from male Sprague-Dawley rats (SasCo., Oklahoma City, OK) were prepared by a modified collagenase perfusion procedure (16). The cells were suspended in medium 1/BSA, and incubated at 37°C for 1 h to increase and stabilize the total cell ASGPR activity (17). The active ASGPRs were purified from hepatocytes by affinity chromatography using ASOR-Sepharose as described previously (18).

Protease treatment. Affinity-purified active ASGPRs were incubated with ASOR-Sepharose in binding buffer (buffer 1 containing 10 mM $CaCl_2$ and 0.05% Triton X-100) at 4°C for 2 h, and the nonbound proteins were removed by washing with the same buffer. The (ASGPR)-ASOR complexes were incubated with binding buffer containing different concentrations of subtilisin A or trypsin at room temperature for 5 min. The supernatant fluids were then transferred into small tubes containing an equal volume of 2-fold concentrated SDS-PAGE sample buffer (19) immediately followed by boiling the sample for 2 min. The ASOR-Sepharose pellets were washed three times with ice-cold binding buffer containing 1 mM PMSF. The bound proteins were eluted with an EGTA-containing buffer as described previously (18), or directly mixed with sample buffer for subsequent electrophoresis.

Production of polyclonal antibody against RHL1 CRD. The RHL1 CRD was obtained by treating ASGPR-ASOR-Sepharose (300 μg ASGPR/ml) with subtilisin A (10 μg/ml) at room temperature for 10 min and subsequent separation by nonreducing SDS-PAGE. The CRD fragment was identified by protein sequencing using the OUHSC Protein Sequencing Facility. The CRD-containing gel band was cut into small pieces, and passed through a 21-gauge needle several times, mixed with an equal volume of Freund's complete adjuvant, and then intradermally injected into a rabbit (0.1 ml/site). Booster injections in Freund's incomplete adjuvant were given two weeks after the first immunization and continued at 3 week intervals. Polyclonal antibodies were obtained by affinity chromatography of total IgG on protein A-Sepharose 4B.

Immunoprecipitation and Western blotting. Cell extracts for immunoprecipitation were prepared from isolated rat as described previously (18). The extract was first incubated with preimmune IgG bound to protein A-Sepharose at 4°C for 2 h to remove nonspecific binding proteins. The supernatant was then incubated at 4°C for 2 h with anti-CRD IgG conjugated to CNBr-activated Sepharose 4B. After centrifugation the supernatants were mixed with equal volumes of 2-fold concentrated sample buffer. The pellets were washed three times with buffer 1 containing 10 mM EGTA and 0.5% Triton X-100, twice with the same buffer containing 0.05% Triton X-100, and then mixed with one volume of sample buffer. For immunoprecipitating inactive receptors, the active receptor from monensin-treated cell extract was depleted by ligand affinity purification prior to immunoprecipitation. Immunopurified proteins were detected by Western blotting using subunit-specific antibodies as described previously (20).

Ligand-binding assays. The ligand-binding activity of purified ASGPRs was determined by a dot blot assay using ^{125}I-ASOR as a ligand (18). Specific ^{125}I-ASOR binding to ASGPRs on isolated hepatocytes was also determined as described (15). To determine the effect of antibody on the ligand-binding activity, cells or nitrocellulose dots (containing 0.1 μg ASGPR) were incubated with antibody at 4°C for 1 h in buffer 1 containing 10 mM EGTA and washed once with binding buffer prior to the incubation with ^{125}I-ASOR.

General. Protein content was determined by the Bradford (21) method, using bovine serum albumin as a standard. SDS-PAGE was performed by the method of Laemmli (19) using 12% crylamide gels. Protein bands were visualized by silver staining or Coomassie Blue R-250.

RESULTS AND DISCUSSION

Previous studies have shown that in purified ASGPRs, the ligand-binding activity of the minor subunits RHL2/3 is more sensitive to some treatments such as reducing agents, denaturing agents or temperature than the major subunit RHL1[4] (13, 18). In addition, we found that the ligand-binding activities of purified RHL1 and RHL2/3 subunits were restored by different detergents after SDS-PAGE and electrotransfer onto nitrocellulose membranes (20). The isolated CRDs of RHL1 and RHL2/3 also display different carbohydrate recognition patterns (13). These observations suggest that the CRDs of RHL1 and RHL2/3 have some regions with different conformations, despite the high extent of identity in their amino acid sequences and the conservation of four disulfide linkages in these do-

[4] F.-Y. Zeng and P. H. Weigel, unpublished results.

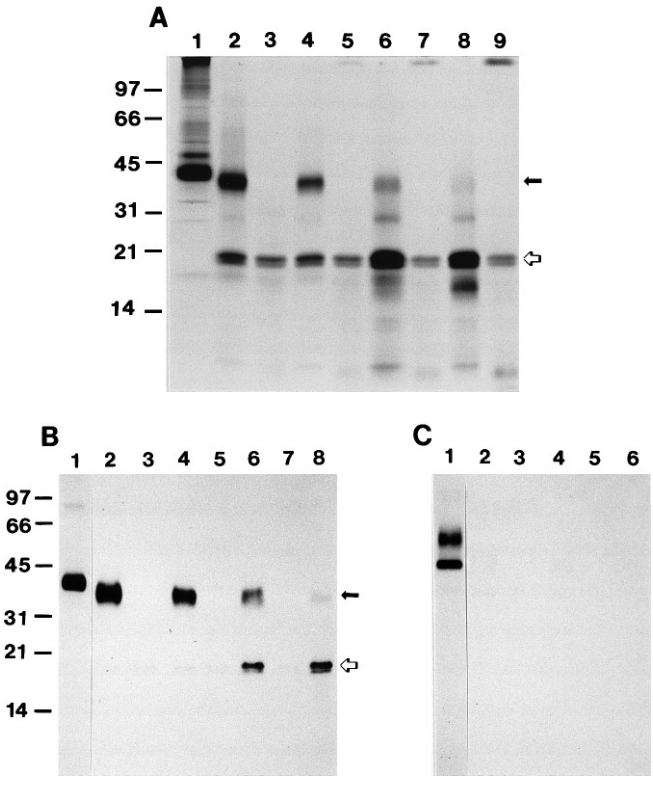

FIG. 1. Generation of RHL1-CRD by subtilisin treatment. Affinity-purified active ASGPRs (2 μg) were bound to ASOR-Sepharose (5 μl) to form ligand-receptor complexes. The complexes were incubated in binding buffer (lane 1) or binding buffer containing subtilisin at an enzyme:receptor ratio (w/w) of 1:200 (lanes 2 and 3), 1:100 (lanes 4 and 5), 1:50 (lanes 6 and 7) or 1:25 (lanes 8 and 9), at room temperature for 5 min. After centrifugation, the supernatant fluids (lanes 3, 5, 7, and 9) were immediately mixed with an equal volume of 2-fold concentrated Laemmli sample buffer (19) and boiled for 2 min. The ASOR-Sepharose pellets (lanes 1, 2, 4, 6, and 8) were immediately washed once with binding buffer containing 1 mM PMSF and then mixed with an equal volume of sample buffer and boiled. The samples were subjected to nonreducing SDS-PAGE. Proteins were visualized by silver staining (A), or by Western blotting using anti-peptide antibodies specific for the C-terminus of RHL1 (B) or RHL2/3 (C). The ~20 kDa CRD is indicated by open arrows and the 40 kDa fragment by solid arrows. The single band in lane B1 is RHL1; the two bands in lane C1 are RHL2 and RHL3.

mains. To address this issue, we examined the differential resistance of RHL1 and RHL2/3 to proteases.

Subtilisin A treatment of ASGPRs immobilized on ASOR-Sepharose in the presence of Ca^{+2} generated two major bands with molecular weights of 40 kDa and 20 kDa on SDS-PAGE in an enzyme dose-dependent manner (Fig. 1). These two fragments retained their ligand-binding ability, since they remained bound to ASOR-Sepharose (Fig. 1A; lanes 2, 4, 6, 8) and they could specifically bind ASOR in a ligand-blot assay after SDS-PAGE (20). In contrast, flow-through fractions contained 20 kDa bands in a relatively constant protein amount (Fig. 1A; lanes 3, 5, 7, 9). The 20 kDa double bands remained unchanged in ASOR-bound fractions when increasing the ratio of enzyme:substrate to 1:25 or more or when prolonging the incubation time up to 1 h (not shown), indicating their resistance to further degradation.

Subunit-specific antibodies that recognize the C-terminus of each RHL CRD were employed to identify from which receptor subunit these fragments were derived. In either the bound or nonbound fractions, RHL2/3-specific antibody (Fig. 1C) recognized none of the protein bands that were readily detected by silver staining (Fig. 1A). In contrast, the RHL1-specific antibody reacted with the 40 kDa band and 20 kDa double bands in the ASOR-Sepharose bound fractions (Fig. 1B). This result indicates that the CRD of RHL1 was resistant to subtilisin treatment, under these conditions, whereas no residual fragments from the RHL2/3 CRDs could be identified. The RHL2/3 subunits appeared to be readily digested into smaller fragments that lost ligand-binding activity. A similar result was also obtained with trypsin (not shown). These findings also support the idea that RHL1 subunits form a ligand-binding core within the hetero-ologomeric receptor, since proteolytic removal of RHL2/3 did not release the RHL1 CRD from ASOR-Sepharose.

In the absence of $CaCl_2$ and ligand, RHL1 was more readily digested into small peptides, and only a low yield of RHL1 CRD could be recovered after subtilisin digestion. Evidently the CRD of RHL1 is more resistant to proteolytic degradation than the CRDs of RHL2 and RHL3 when bound to ASOR in the presence of Ca^{+2}. These results indicate that the CRD conformations of RHL1 and RHL2/3 may be different in spite of their high sequence homology. The RHL1 CRD may possess a conformation that is more compact and inaccessible. The presence of $CaCl_2$ and ligand is required to maintain this conformation, since their absence essentially eliminated recovery of the RHL1 CRD (not shown). For the analogous chicken ASGPR it was also found that $CaCl_2$ can protect the CRD from enzyme digestion (22).

N-terminal sequence analysis confirmed that both the 40 kDa and 20 kDa fragments were derived from RHL1. The 20 kDa double bands showed two N-termini representing cleavage at Met^{101}-Lys^{102} and Leu^{115}-Arg^{116} of RHL1 (4). Both 20 kDa fragments contained the intact CRD of RHL1, since they reacted with antibody specific for the C-terminus. We then produced a rabbit polyclonal antibody against the SDS-PAGE purified, nonreduced RHL1 CRD. To our surprise, Western blot analysis revealed that this antibody could recognize only RHL1 either in crude rat hepatocyte extracts or in affinity-purified ASGPRs under reducing or nonreducing conditions (Fig. 2). The specificity of the antibody was very high, since no detectable reactivity was observed with RHL2/3. No immunoreactivity was seen with the preimmune serum or in the absence of primary antibody (not shown).

Immunoprecipitation with the anti-RHL1 CRD anti-

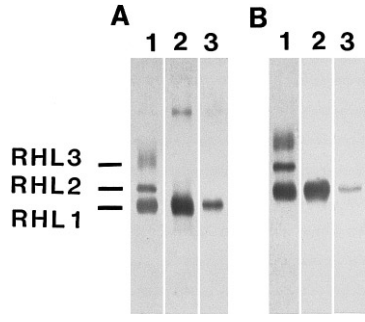

FIG. 2. Reactivity of anti RHL1-CRD antibody with rat ASGPRs. Affinity purified ASGP receptors (lanes 1 and 2) and hepatocyte extract (lane 3) were subjected to SDS-PAGE under nonreducing (A) and reducing (B) conditions, and electrotransferred onto a nitrocellulose membrane. The nitrocellulose strips were incubated with a mixture of anti-RHL1 and anti-RHL2/3 antibodies (lane 1) or anti-RHL1 CRD antibody (lanes 2 and 3) and processed for Western analysis as described in Methods.

body showed co-purification of RHL2 and RHL3 from both total cell extracts and affinity-purified ASGPRs (Fig. 3). The antibody quantitatively removed all receptor subunits from the extract (Fig. 3, lanes 4p and 4s). This result confirms that active receptors are heterooliomers with no homo-oligomers of RHL2/3, at least after detergent solubilization. The patterns of immunopurified receptors and ligand affinity-purified active receptors were nearly identical. Based on densitometry of lanes 4p, 5p and 6p, the approximate subunit stoichiometry of antibody-purified receptors from total extract was 2.9:1.3:1.0, (RHL1:RHL2:RHL3) and that for active receptors purified by ASOR-Sepharose was 2.8:0.9:1.0 (RHLl:RHL2:RHL3, lane 1). Therefore, there is no evidence for a substantial pool of homo-oligomeric RHL2/3 receptors in hepatocytes.

Although the anti-RHL1 CRD antibody failed to recognize RHL2/3 on Western blots, it nonetheless inhib-

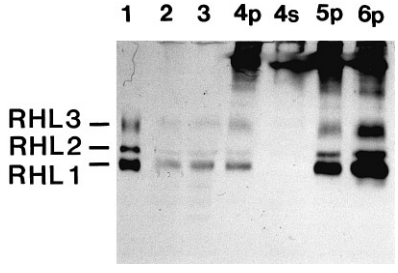

FIG. 3. Immunoprecipitation of RHL2/3 and RHL1 with anti-RHL1 CRD antibody. ASGPRs were immunoprecipitated from total cell extract [lanes 4 (25 μg), 5 (100 μg) and 6 (250 μg)] using the anti-RHL1 CRD antibody as described under Methods. The supernatant fluids (designated s) and the Sepharose pellets (designated p) were subjected to nonreducing SDS-PAGE and Western blotting using a mixture of anti-RHL1 and anti-RHL2/3 antibodies. For comparison, affinity-purified receptor (lane 1, 25 ng) and total cell extract (lane 2, 12.5 μg; lane 3, 25 μg), are also shown. The major band at the top of lanes 4-6 is IgG.

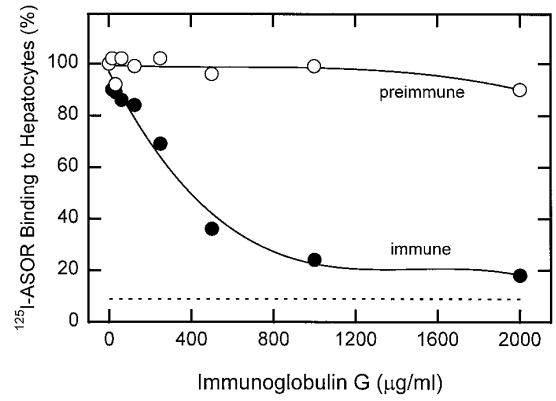

FIG. 4. Effect of anti-RHL1 CRD antibody on ^{125}I-ASOR binding to hepatocytes. Isolated hepatocytes were permeabilized with 0.055% digitonin, washed and then incubated at 4°C for 1 h in Medium 1/BSA (15) containing 10 mM EGTA and the indicated concentrations of preimmune (○) or anti-CRD (●) IgG. The cells were then washed and ^{125}I-ASOR binding was assessed in Medium 1/BSA as described previously (15,17). Values are the average of duplicates. Nonspecific binding, determined in the presence of excess ASOR, was ~5%.

ited ^{125}I-ASOR binding to isolated rat hepatocytes in a dose-dependent manner (Fig. 4). About 80-90% of ^{125}I-ASOR-binding activity to cell surface ASGPRs (not shown) or to total cellular ASGPRs (surface plus intracellular) could be blocked by this antibody (Fig. 4). At least 90% of the ligand-binding activity of purified rat ASGPRs was also inhibited, as assessed in a dot blot assay (Fig. 5). No inhibition of ^{125}I-ASOR binding to hepatocytes was observed with nonimmune IgG, or antibodies to the C-terminal peptides of either RHL2/3 or RHL1 (not shown). In an earlier study, a polyclonal

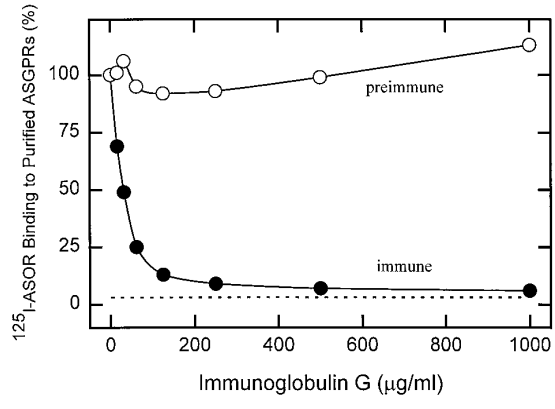

FIG. 5. Effect of anti-RHL1 CRD antibody on ^{125}I-ASOR binding to affinity-purified ASGPRs. Rat ASGPRs, affinity-purified using ASOR-Sepharose, were immobilized onto nitrocellulose (0.1 μg/dot) using a dot blot apparatus. After blocking with 0.1% Tween 20 in Buffer 1 containing 10 mM $CaCl_2$ (BIC10) at 4°C for 1 h the dots were incubated with the indicated concentrations of preimmune (○) or anti-CRD (●) IgG in PBS containing 0.1% Tween 20 for 1 h at 4°C. The dots were then washed, incubated with 0.75 μg/ml ^{125}I-ASOR in BIC10 with 0.02% Tween 20 at 4°C for 1 h, washed again and bound radioactivity was determined. Nonspecific binding was ~2%.

antibody to the total RHL2/3 subunits was able to block ligand binding (23). All of these results are consistent with the conclusion that, both in cellular membranes and after detergent solubilization, functional ASGPRs able to bind ASOR are hetero-oligomers, and that the minor subunits (RHL2/3) are required for the high affinity ligand-binding.

Although coexpression of both HL subunits and assembly of a hetero-oligomer is required to get stable expression of the minor subunit (RHL2/3 or HHL2) and its appearance at the cell surface, there has been no evidence to exclude the possibility that subsequent subunit rearrangement could give rise to stable and functional homo-oligomers of RHL2/3 and/or RHL1. The report of these crosslinked species (4) could support the presence of RHL2/3 homo-oligomers. Based on the present study, however, this possibility is unlikely. Although homo-oligomeric RHL1 or HHL1 can bind a typical ligand such as ASOR with lower affinity (9), we conclude that homo-oligomeric RHL 2/3 able to bind ASOR is not generated on the cell surface by rearrangement of RHL subunits.

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