Second Messenger Modulation of the Asialoglycoprotein Receptor*

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Post-transcriptional regulation of the asialoglycoprotein receptor (ASGR) in the HepG2 cell line can be mediated by the presence of biotin in the culture medium. To determine if the induction by biotin of intracellular cGMP affects ASGR expression, HepG2 were grown in biotin-depleted medium with the cell-permeant 8-bromo-cGMP (8-Br-cGMP). Both cell-surface and total ASGR binding of radiolabeled asialoorosomucoid (125I)-ASOR increased from 30 to 95% of control levels by the addition of increasing concentrations of 8-Br-cGMP. The rate of ASGR-mediated endocytosis of 125I-ASOR also increased with increasing concentrations of 8-Br-cGMP. Estimates of the steady state levels of ASGR by transblot analysis utilizing both antisera to affinity-purified ASGR and to isoform-specific antibodies prepared against synthetic peptides confirmed that the increase in 125I-ASOR binding was due to an increase in ASGR expression. Metabolic labeling of biotin-deprived HepG2 with [35S]cysteine and sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of immunoprecipitates revealed an increase of radiolabeled ASGR within 30 min of the addition of 8-Br-cGMP. Induction of cGMP by atrial natriuretic factor also increased the metabolic labeling of ASGR. ASGR expression in a second hepatocellular carcinoma cell line, HuH-7, responded in a similar fashion to the addition of 8-Br-cGMP. In contrast to 8-Br-cGMP, exposure to 8-bromo-cAMP results in a reduction of ASGR expression even in the presence of biotin-containing medium. The antagonistic roles of cGMP and cAMP suggest a balance between cyclic nucleotides is required for the maintenance of differentiated functions by the hepatocyte.

Expression of the asialoglycoprotein receptor (ASGR)* responsible for the endocytosis of galactose/N-acetylgalactosamine terminating glycoproteins (for review see Refs. 1-4) is characteristic of fully differentiated hepatocytes. Detection of ASGR first becomes apparent late in primary development

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§ The abbreviations used are: ASGR, asialoglycoprotein receptor; 8-Br-cGMP, 8-bromo-cGMP; 8-bromoguanosine 3’5’-cyclic monophosphate; 8-Br-cAMP, 8-bromoadenosine 3’5’-cyclic monophosphate; ASOR, asialoorosomucoid; ANP, atrial natriuretic factor; MEM, minimal essential medium; FBS, fetal bovine serum; dFBS, dialyzed fetal bovine serum; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; FITC, [ethylendioxythylamino]-[8-tetracyano-9-phenyl]propionic acid (8-Br-cGMP). Both cell-surface and total ASGR binding of radiolabeled asialoorosomucoid (125I)-ASOR was increased from 30 to 95% of control levels by the addition of increasing concentrations of 8-Br-cGMP. The rate of ASGR-mediated endocytosis of 125I-ASOR also increased with increasing concentrations of 8-Br-cGMP. The 3-fold increase of both intracellular and cell-surface ASGR that occurs during pregnancy followed by a precipitous drop at parturition (6) is strongly suggestive that receptor expression is also under hormonal control. In support of hormonal control is the observation that dexamethasone reduces the amount of ASGR in the liver of newborn rats (16). Removal of the low molecular weight components of fetal bovine serum (FBS) medium supplement by dialysis (dFBS) dramatically reduces ASGR expression in HepG2 without a concomitant reduction in receptor-specific mRNA (17, 18). However, the addition of 1 nM biotin not usually considered a hormone to dFBS restores full expression of ASGR with ligand-binding characteristics and molecular mass comparable to receptor isolated from human liver (18). Biotin induces the activity of glucokinase and the intracellular levels of cGMP in primary cultures of adult rat hepatocytes (19, 20). The effects of biotin upon enzyme activity were mimicked by the addition of the cell-permeant 8-bromo-cGMP. In the present study, we observed a direct link between cGMP and ASGR expression by HepG2 and a second hepatocarcinoma cell line, HuH-7.

MATERIALS AND METHODS

Human asialoorosomucoid (ASOR) was prepared by neuraminidase (Genzyme) treatment as previously described (21). ASOR and Protein A (Sigma) were iodinated by the solid phase lactoperoxidase-glucose oxidase-coupled procedure as described by the manufacturer (Enzymoabads, Bio-Rad). Two 16-amino acid peptides, one corresponding to the carboxyl terminus (TCETLDKASQEPPLL) of the predominant (H1) receptor subunit and a unique sequence (GEGPGTRRLNPRRGNP) near the amino end of the minor (H2) subunit (22), were synthesized by solid phase at the Laboratory of Macromolecular Analysis, Albert Einstein College of Medicine. Other materials were obtained from the following: carrier-free 35S and 85S cysteine (specific radioactivity of >600 Ci/mol); Amersham Corp.; molecular weight standards, Bethesda Research Laboratories; nitrocellulose filters, Schleicher and Schuell Inc.; keyhole limpet hemocyanin, anti-rabbit IgG-horseradish peroxidase, Calbiochem Behring Corp.; GammaBind G-Agarose, Genex; dimethyl 3,5-dithiobis(propionimidate), Pierce Chemical Co.; 8-bromoguanosine 3’5’-cyclic

monophosphate, (8-Br-cGMP), 8-bromoadenosine 3′,5′-cyclic monophosphate (8-Br-cAMP), atrial natriuretic factor (ANF) peptide, Freund's complete and incomplete adjuvant, "Sigma"; fetal bovine serum, dialyzed fetal bovine serum, Eagle's minimal essential medium, penicillin-streptomycin solution, Gibco Laboratories; seromucoid 96-well EIA plate, Costar. All other chemicals were reagent grade.

**Cell Culture—**HepG2 and HuH-7 were plated in 30-mm dishes (Falcon), allowed to reach confluence in minimal essential medium (MEM), 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 μg/ml streptomycin. Medium was changed to MEM supplemented with dFBS in experimental dishes or with FBS in controls. Cells were grown near to or at confluence before each experiment. Protein was determined by the Lowry procedure (23) and used to normalize the data.

**Binding of ASOR to HepG2—**Cells were preincubated for 1 h in serum-free MEM made 5 mM in CaCl₂ (MEM-CA) at 37 °C. To assay surface binding, cells were chilled to 4 °C and incubated for 1 h with 35S-[2] added to 1 mg/ml of MEM-CA containing 1 mg/ml bovine serum albumin. Nonspecific ligand binding was estimated from culture dishes that also contained 100 μg of unlabelled ASOR. Unbound ASOR was removed by two washes with 1.5 ml of MEM-CA and a final wash with MEM-CA made 0.5 mM in N-acetyl-D-galactosamine (GalNAc). Surface-bound ASOR was released with 50 mM GalNAc. Total ASOR binding was determined in cells following exposure for 30 min at 4 °C to 0.1% bovine serum albumin to which an equal volume of 20% trichloroacetic acid, 4% phosphotungstic acid was added. The precipitated protein was collected on a nitrocellulose paper and detected with antibody and iodinated protein A as described by Towbin et al. (28).

**Endocytosis of Surface-bound ASOR—**The rate of internalization of surface-bound ASOR was estimated as previously described (25). Briefly, HepG2 surface receptors were saturated with ASOR at 4 °C as above, and the cells shifted to 37 °C. At various times, cells were cooled to 4 °C, and an aliquot of medium was added to an equal volume of 20% trichloroacetic acid, 4% phosphotungstic acid to assess ligand degradation estimated by acid-soluble radioactivity. The cells were washed twice with MEM-CA and incubated in 50 mM GalNAc to remove residual ASOR. The surface-bound ASOR that was internalized was estimated as the fraction resistant to GalNAc release.

**Anti-ASOR Antibodies—**Rabbit antibodies to the ASOR isolated from human liver have been previously described (17, 18). Peptides were linked to keyhole limpet hemocyanin, using the chemical cross-linking agent dimethyl 3,3′-dithiodi(propionimidate). Equal amounts (1 mg) of peptide and hemocyanin were dissolved in 1.0 ml of 0.05 M phosphate-buffered saline, pH 7.4, to which 20 mg of dimethyl 3,3′-dithiodi(propionimidate) was added with rapid mixing. The conjugate was allowed to proceed for 30 min at 22 °C, after which the conjugate mixture was added to complete Freund's adjuvant. Rabbits were immunized, then boosted every 2 to 3 weeks with 50 μg of hemocyanin-peptide in incomplete Freund's adjuvant. The specificity of the antisera was assayed by direct enzyme-linked immunosorbent assay against 100 ng of peptide per well (26).

**Biosynthetic Labeling—**Cells were preincubated in MEM minus cysteine containing 10% dFBS for 30 min at 37 °C. Metabolic labeling was performed with 35S-cysteine, 200 μCi/ml for 10 min. Cells were harvested, washed with ice cold 0.05 M phosphate-buffered saline, pH 7.4, pelleted at top speed in an Eppendorf centrifuge, and stored at −70 °C until used.

**Immunoprecipitation of ASOR—**[35S]Cysteine-labeled cells were defrosted and lysed in 1 ml of lysis buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 0.1% bovine serum albumin, 1% Nonidet P-40, 1 mM EGTA, 2 mM phenylmethylsulfonyl fluoride containing 100 units of aprotinin, 1 μg of leupeptin, 1 μg of pepstatin. To estimate the amount of [35S]cysteine incorporated into protein, a 50 μl aliquot of the lysate was added to 0.5 ml of 10 mM Tris, pH 7.4, 150 mM NaCl containing 0.1% bovine serum albumin to which an equal volume of 20% trichloroacetic acid was added. The precipitated protein was collected on a Whatman GF/A glass fiber filter under reduced pressure and washed with 10 ml of methanol. The air-dried filter was dispersed in 10 ml of Hydroflour (National Diagnostics), and the amount of 35S was measured by liquid scintillation counting. Aliquots of cell lysates containing an equal amount of radiolabeled protein were precloned by the addition of 20 μl of Gammabond G (50% suspension) prebound with 10 μl of preimmune serum and incubation at 4 °C with constant mixing for 30 min. The lysates were centrifuged, and 10 μl of polyclonal antisera and 50 μl of Gammabond G were added to the supernatant. Following 30 min incubation at 4 °C with constant mixing, the lysates were centrifuged, and the pellets were washed with 10 ml Tris, 150 mM NaCl, 1% Triton X-100, 1% deoxycholate, and 0.1% SDS, pH 7.4, three times and a final wash with 50 mM Tris-Cl, 150 mM NaCl, pH 7.4. An equal volume of 2× SDS-PAGE sample buffer was added, and the pellets were heated at 90 °C for 10 min prior to electrophoresis on a 10% gel (27). The fixed gel was prepared for fluorography with ENHANCE (Du Pont-New England Nuclear) and exposed to Kodak SB-5 film at −70 °C.

**Immunoblotting—**Aliquots of cell lysate in SDS-PAGE sample buffer were heated at 90 °C for 10 min before resolution of the proteins on a 10% gel (27). Proteins were electrophoretically transferred to nitrocellulose paper and detected with antibody and iodinated protein A as described by Towbin et al. (28).

**RESULTS**

HepG2 grown to confluence in MEM supplemented with FBS bound a total of 30 ± 1.2 ng of 125I-ASOR/mg of cell protein in detergent permeabilized preparations (Fig. 1). Of this binding activity, 10.2 ± 0.2 ng of 125I-ASOR/mg of protein can be assigned to cell-surface ASOR and 19.8 ± 0.3 ng of 125I-ASOR/mg of protein to the intracellular pool of ASOR. Cells grown in medium supplemented with dFBS expressed only 30% of the control activity, while the ratio of total to cell-surface binding activity remains unaltered (Fig. 1). The addition of cell-permeant 8-Br-cGMP to cells grown in medium containing dFBS for 48 h before assay increased the amount of ASOR bound in a concentration-dependent fashion (Fig. 1). Both the cell surface and total amount of ASOR bound increased, reaching 95% of control at 10 mM 8-Br-cGMP. Cells grown in 20 mM 8-Br-cGMP or for up to 96 h in 10 mM 8-Br-cGMP did not exhibit a further increase of binding activity. The binding activity of control cells was not increased by supplementing the growth medium with up to 20 mM 8-Br-cGMP.

The rate of ASOR-mediated endocytosis in cells grown in MEM supplemented with FBS or dFBS containing increasing concentrations of 8-Br-cGMP was estimated by the amount of cell-surface bound ASOR internalized (Fig. 2) and degraded (Fig. 3). Cells grown in FBS-containing medium internalized 1.5 ng of ASOR/min/mg of protein over the first 5 min, while those grown in dFBS-containing medium internalized 0.4 ng of ASOR/min/mg of protein. The addition of 10 mM 8-Br-cGMP restored the internalization rate to 1.4 ng of ASOR/min/mg of protein (Fig. 2). As was the case for binding activity, the addition of higher concentrations of 8-Br-cGMP did not result in an increase of the internalization rate. In all cases, degradation products of internalized ASOR were detected in the culture medium after a 15-min lag period, and

![Fig. 1. Influence of 8-Br-cGMP on the specific binding of 125I-ASOR to HepG2 grown in MEM supplemented with dFBS.](http://www.jbc.org/). Cells were grown to near confluence in media with either 10% FBS or dFBS (C) to which increasing concentrations of 8-Br-cGMP were added 48 h before binding assays were performed as described under "Materials and Methods." The results are the mean ± S.E.M. of triplicate determinations.
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FIG. 2. Effects of 8-Br-cGMP on the rate of 125I-ASOR internalization by HepG2 grown in MEM supplemented with dFBS. Cells were grown to near confluence in media with either 10% FBS (○), dFBS (△), or dFBS made 10 mM with 8-Br-cGMP (▼) 48 h before assays were performed as described under “Materials and Methods.” The results are the means of duplicate determinations.

FIG. 3. Effect of 8-Br-cGMP on the degradation rate of 125I-ASOR by HepG2 grown in MEM supplemented with dFBS. Cells were grown to near confluence in media with either 10% FBS (○), dFBS (△), or dFBS made 10 mM with 8-Br-cGMP (▼) 48 h before assays were performed as described under “Materials and Methods.” The results are the means of duplicate determinations.

The amount of acid-soluble radioactivity was proportional to the amount of ASOR internalized (Fig. 3).

The steady state concentration of ASGR estimated by transblot analysis using a polyclonal antibody raised against the affinity-purified human ASGR confirmed that the loss of binding activity and its restoration were due to changes in the amount of protein expressed (Fig. 4). HepG2 grown in MEM supplemented with dFBS and at least 1 mM 8-Br-cGMP showed an increase of ASGR over the basal level and, when grown in 10 mM 8-Br-cGMP, closely approximated the amount of metabolically labeled ASGR in HepG2 grown to confluence in MEM supplemented with dFBS (Fig. 6). The level of radioactivity labeled ASGR exhibited by control cells maintained in MEM plus FBS was achieved in the 8-Br-cGMP-treated cells following 24 h of exposure to the cyclic nucleotide. Consistent with our previous observation that biotin deprivation did not significantly reduce protein synthesis (17), there was no change in the amount of trichloroacetic acid-precipitable radioactivity in cell lysates over the 24-h period of 8-Br-cGMP treatment (data not shown).

Atrial natriuretic factor (ANF) has been shown to selectively activate the particulate guanylate cyclase and elevate cGMP in rat liver (30). HepG2 were grown to confluence in MEM plus dFBS and treated with 1 μM ANF peptide for 30 min prior to metabolic labeling with [35S]cysteine. Densito-
Fig. 6. Induction of metabolically labeled ASGR by 8-Br-cGMP in HepG2. Cells were grown to confluence in media supplemented with 10% FBS or 10% dFBS (a) to which 0 mM 8-Br-cGMP or 5 mM 8-Br-cGMP (b) was added for: 10 min (c), 30 min (d), 1 h (e), 5 h (f), 24 h (g). Cells were washed with MEM minus cysteine plus 10% dFBS labeled in the same media with [35S]cysteine, and the ASGR immunoprecipitates were processed as described under “Materials and Methods.”

Fig. 7. Induction of metabolically labeled ASGR by atrial natriuretic factor (ANF). Cells were grown to confluence in media supplemented with 10% FBS (a) or 10% dFBS (b) to which 1 mM ANF peptide (c) was added 30 min before [35S]cysteine labeling as described in Fig. 6.

Fig. 8. Effect of 8-Br-cGMP on the expression of ASGR in HuH-7. Cells were grown to near confluence in media supplemented with either 10% FBS (a), 10% dFBS containing 10 mM 8-Br-cGMP (b) or 10% dFBS alone (c) for 48 h before harvesting. Cells were lysed, and 25 μg of protein was resolved by SDS-PAGE for transblot analysis as described under “Materials and Methods” and in Fig. 4.

Fig. 9. Effect of 8-Br-cAMP on the expression of ASGR in HepG2. Cells were grown to near confluence in media containing either 10% FBS (a), 10% dFBS (b), 10% FBS plus 10 mM 8-Br-cAMP (c), 10% FBS plus 10 mM 8-Br-cGMP (d), 10% dFBS plus 10 mM 8-Br-cAMP (e), or 10% dFBS plus 10 mM 8-Br-cGMP (f) for 48 h before harvesting. Cells were lysed, and 25 μg of protein per well was resolved by SDS-PAGE for transblot analysis as described under “Materials and Methods” and in Fig. 4.

Fig. 10. Influence of 8-Br-cAMP on the specific binding of 125I-ASOR to HepG2 grown in media supplemented with 10% FBS. Cells were grown to near confluence in media supplemented with 10% FBS to which increasing concentrations of 8-Br-cAMP were added 48 h before binding assays were performed as described under “Materials and Methods.” The results are the mean ± S.E.M. of triplicate determinations.

cGMP to the growth media restored ASGR to near control level (Fig. 8). In contrast to the induction of ASGR promoted by 8-Br-

metric scanning of the autoradiogram indicates a 6.0-fold increase in immunoprecipitable radiolabeled ASGR in the ANF-treated cells as compared to cells grown in MEM supplemented with dFBS alone (Fig. 7). This amount of radiolabeled ASGR was equivalent to 36% of that recovered from cells grown in MEM containing 10% FBS.

A second human hepatoma cell line, HuH-7, established from a hepatocellular carcinoma (31), expresses the ASGR in amounts equivalent to that seen in confluent HepG2 (Fig. 8). HuH-7 maximally expressed ASGR when cells were grown to confluence in MEM plus 10% FBS. Consistent with the results obtained with HepG2, HuH-7 grown in medium plus dFBS showed a reduced level of ASGR. The addition of 8-Br-

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a b c d e f g

200 97 43 29

43 29

Fig. 6. Induction of metabolically labeled ASGR by 8-Br-cGMP in HepG2. Cells were grown to confluence in media supplemented with 10% FBS or 10% dFBS (a) to which 0 mM 8-Br-cGMP or 5 mM 8-Br-cGMP (b) was added for: 10 min (c), 30 min (d), 1 h (e), 5 h (f), 24 h (g). Cells were washed with MEM minus cysteine plus 10% dFBS labeled in the same media with [35S]cysteine, and the ASGR immunoprecipitates were processed as described under “Materials and Methods.”

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Fig. 8. Effect of 8-Br-cGMP on the expression of ASGR in HuH-7. Cells were grown to near confluence in media supplemented with either 10% FBS (a), 10% dFBS containing 10 mM 8-Br-cGMP (b) or 10% dFBS alone (c) for 48 h before harvesting. Cells were lysed, and 25 μg of protein was resolved by SDS-PAGE for transblot analysis as described under “Materials and Methods” and in Fig. 4.

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Fig. 10. Influence of 8-Br-cAMP on the specific binding of 125I-ASOR to HepG2 grown in media supplemented with 10% FBS. Cells were grown to near confluence in media supplemented with 10% FBS to which increasing concentrations of 8-Br-cAMP were added 48 h before binding assays were performed as described under “Materials and Methods.” The results are the mean ± S.E.M. of triplicate determinations.

cGMP to the growth media restored ASGR to near control level (Fig. 8). In contrast to the induction of ASGR promoted by 8-Br-
cGMP, treatment of HepG2 with 8-Br-cAMP inhibits receptor expression. The steady state concentration of ASGR in HepG2 grown to confluence in MEM supplemented with 10% FBS containing 10 mM 8-Br-cAMP is reduced to a level approximately equivalent to that seen in cells grown in MEM containing dFBS (Fig. 9). Addition of 10 mM 8-Br-cAMP to MEM plus dFBS further reduces the steady state concentration of ASGR in cells grown to confluence in this medium to below the level of immunodetection (Fig. 9). A loss of cell surface and total ASGR binding activity paralleled the loss of protein expression (Figs. 10 and 11) with no specific binding of ASOR being evident in cells grown in medium supplemented with dFBS to which 10 mM 8-Br-cAMP was added (data not shown).

**DISCUSSION**

Biotin has been established as a requirement for the maximal expression of ASGR (18) and most probably the insulin receptor (17) by HepG2 cells. The addition of the vitamin to tissue homogenates has been reported to increase the activity of guanylate cyclase (20), and, when added to primary cultures of adult rat hepatocytes, the intracellular level of cGMP rose rapidly (19), suggesting a potential role for cGMP in the regulation of ASGR expression.

When HepG2 were grown to confluence in MEM supplemented with dFBS containing increasing concentrations of 8-Br-cGMP, both cell surface and total ASGR binding activities were increased (Fig. 1). Measurement of ASGR endocytotic activity, i.e. ligand internalization and degradation (Figs. 2 and 3), indicated that both the functional and nonfunctional or silent fractions (32, 33) of cell-surface ASGR are equally affected. These parallel increases suggest that cGMP does not alter ASGR distribution as has been reported for phorbol ester treatment of HepG2 (24) and the plasma membrane remodeling resulting during hepatic regeneration (11-13). Rather, the effect of cGMP on ASGR expression appears similar to the global induction seen during hepatocellular development or the dramatic 3-fold increase of ASGR activity which occurs during pregnancy (5-7).

The direct correlation between the steady state level of ASGR as estimated by transblot analysis and the concentration of 8-Br-cGMP added to biotin-deprived HepG2 (Fig. 4) was consistent with the observed response in ASGR activity (Figs. 1–3). Absence of a super ASGR induction by the addition of 20 mM 8-Br-cGMP or by a longer exposure to the cyclic nucleotide (data not shown) supports the proposal that the effective concentration of biotin in FBS is sufficient to maximally activate guanylate cyclase (34). When peptide-specific antibodies were used as probes of ASGR concentrations (Fig. 5), it became evident that HepG2 requires the presence of cGMP to express both the major (H1) and minor (H2) isoforms of the protein (22). This is to be expected in the light of the report that co-transfection of both ASGR genes is required to attain endocytic activity (35-37).

**REFERENCES**


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