Posttranscriptional Regulation of the Asialoglycoprotein Receptor by cGMP*

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The human asialoglycoprotein receptor expressed by the HepG2 cell line is composed of the two homologous polypeptides H1 and H2. Transblot analysis of HepG2 cell lysates indicated that the progressive loss in the steady-state level of asialoglycoprotein receptor (ASGR) when cells were maintained in medium supplemented with dialyzed fetal bovine serum was reversed by the addition of cell-permeant 8-bromo-cGMP. Estimates of the steady-state levels of H1- and H2-related mRNA by Northern blot analysis indicated that the reduction of ASGR was not the result of a concomitant reduction in gene transcript number. No difference in the translatability of the mRNAs derived from cells grown in medium supplemented with fetal bovine serum or its dialyzed counterpart was detected. Resolution of the mRNAs by sucrose gradient centrifugation suggests that cGMP-mediated posttranscriptional regulation of ASGR expression was due to a shift of both H1 and H2 mRNAs from the ribonucleoprotein fraction into a translationally active membrane-associated polysomal pool.

The human asialoglycoprotein receptor (ASGR) is responsible for the endocytosis of galactose and/or N-acetylgalactosamineterminating glycoproteins by the parenchymal cell of mammalian liver and by the well differentiated human blastoma cell line, HepG2 (for review see Refs. 1–4). 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 marked effect on total protein synthesis (5). The addition of 1 nM biotin to dFBS restores full expression of ASGR with ligand-binding characteristics and molecular mass comparable with receptor isolated from human liver. The proposed mechanism of biotin’s regulation of ASGR expression by increasing the level of intracellular cGMP via the activation of guanylate cyclase (6, 7) was supported by the induction of ASGR expression following the addition of the cell-permeant 8-bromo-cGMP to the culture media of HepG2 and to a second liver cell carcinoma line HUH-7 (8). Enhancement of ASGR synthesis in HepG2 by atrial natriuretic factor, a known activator of the particulate guanylate cyclase (9), provided independent evidence that intracellular concentrations of cGMP regulate the expression of ASGR at the biosynthetic level (8).

The results of the present study suggest that the effect of cGMP was at a posttranscriptional level as the result of translocation of ASGR mRNA to a membrane-associated pool.

Materials and Methods

Cell Culture—HepG2 were plated in 30-mm dishes (Falcon) from confluent cultures in minimal essential medium (MEM), 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin. Medium was changed to MEM supplemented with FBS, dFBS, or dFBS plus 8-bromo-cGMP (Sigma). Protein was determined by the Lowry procedure (10) and used to normalize the data.

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 (11). Proteins were electrophoretically transferred to nitrocellulose paper, and total ASGR was detected with a polyclonal rabbit antibody recognizing both H1 and H2 subunits (8) and iodinated protein A as described by Towbin et al. (12). Autoradiographs were quantified using an Ultrascan XL densitometer (Pharmacia LKB Biotechnology Inc.).

RNA Isolation and Analysis—Total cytoplasmic RNA was isolated by extraction with guanidine thiocyanate (13) from approximately 106 HepG2 cells. Polyadenylated RNA was isolated using an oligo(dT)-cellulose column, (poly(A) Quik, Stratagene) as described by the manufacturer.

Polysome-associated RNA was isolated within a sucrose gradient as described by Atkinson et al. (14) with the addition of 150 μg/ml cycloheximide, 0.5 μg heparin sulfate, and 100 units/ml RNasin (Promega Biotech) to the homogenization buffer. Fractions containing the membrane-associated polysomes were diluted 3-fold with homogenization buffer and centrifuged at 60,000 × g for 1 h. RNA was isolated from the pellet by guanidine thiocyanate extraction (13).

Polysome gradients were prepared from a postmitochondrial supernatant (24,000 × g for 15 min) of cell homogenates. Aliquots were layered over 15–50% linear sucrose gradients diluted with the homogenization buffer (20 mM HEPES, pH 7.5, 250 mM sucrose, 100 mM KCl, 5 mM MgCl2, 50 μg/ml cycloheximide, and 5 mM dithiothreitol) containing 100 units/ml RNasin. Gradients were centrifuged for 8.5 h at 4 °C in an SW 28 rotor at 85,000 × g. Polysome profiles were recorded by monitoring UV absorption at 260 nm, and gradients were collected in 10 fractions. RNA was precipitated from each fraction by the addition of 0.1 volume of 3 M sodium acetate, followed by 2.5 volumes of ice-cold ethanol and storage overnight at −20 °C. RNA was prepared from the ethanol precipitates by extraction with guanidine thiocyanate, major isoform.

For Northern blot analysis, RNA samples were electrophoresed on horizontal denaturing formaldehyde-agarose gels and transferred to a Nytran membrane (Schleicher and Schuell) under vacuum. For slot-blot analysis, recovered RNA was dissolved in 0.1 ml of 10 ×...
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SSC (1.0 × SSC: 8.75 g of NaCl, 4.41 g of sodium citrate/liter, pH adjusted to 7.0 with 10 N NaOH) heated to 65 °C for 10 min and transferred to a nitrocellulose filter (Schleicher and Schuell) under vacuum. Probes were random prime-labeled (Amersham Corp.) at 37 °C for 1 h to a specific activity of 1–5 × 10⁸ cpm/μg of DNA. Hybridization was performed using standard techniques (15), with the addition of a higher stringency wash at 60 °C in 15 mM NaCl, 1.5 mM Na₂HPO₄, 0.2 M NaOH, pH 7.0, 0.1% SDS for 1 h. In some cases, previously hybridized probes were removed for rehybridization of the blots. Equal loading and blotting of RNA samples was verified by staining the membranes with methylene blue (17).

Probes for Hybridization—A full-length cDNA encoding the minor form of the human asialoglycoprotein receptor, H2, was cloned from a rabbit ASGR cDNA library (Clontech Laboratories, Inc.) by screening with a rat ASGR cDNA probe for RHL-1, provided by Dr. K. Drickamer, Columbia University. For probing of Northern blots, a 260-base pair fragment was prepared from the full-length H2 cDNA clone by sequential restriction nuclease digestions (SacI and HindIII); it encompasses base pairs 155–415 of the H2 sequence published by Spiess and Lodish (18) and covers a 57-nucleotide region (base pairs 187–249) previously found to be unique to H2 relative to H1.

Several lines of evidence have demonstrated that H1 and H2 polypeptides interact early in biosynthesis and that full expression of H2 is dependent upon the presence of H1 (19–25). To determine whether the reduction of both H1 and H2 polypeptides was the result of an alteration in protein interactions or reflected a more direct effect on the two independent gene transcripts, the levels of expression of H1 and H2 mRNAs were determined. Northern blot analysis of mRNA using the H1 and H2 probes indicated a small but reproducible difference in the molecular mass of the transcript (Fig. 2). The message detected by the H2-related probe corresponds to the approximately 1.5-kilobase transcript previously reported for H2 in HepG2 cells (18). When the H2 probe was used in the selection of clones from a HepG2 library, sequencing of positive clones indicated that only H2 inserts were obtained (data not shown). Based on these results, the H1 and H2 probes were used to define the level of expression of the two mRNAs.

When HepG2 cells were maintained in MEM supplemented with dFBS, no difference in the abundance of H1- or H2-specific mRNA, as compared with control cells in MEM plus 10% FBS, could be detected (Fig. 3). Maintenance of the steady-state concentration of the ASGR in HepG2 by the addition of 8-bromo-cGMP to MEM plus dFBS also was without effect on the steady-state concentrations of H1- or H2-specific mRNAs (Fig. 3).

The translatability of the ASGR H1 subunit mRNA isolated from biotin-deprived HepG2 cells was assessed in a cell-free system. In vitro translation of the isolated mRNA directed the synthesis of a polypeptide with the expected molecular mass of 34 kDa (26) (Fig. 4). A comparison of the immuno-recovered polypeptides translated from equal amounts of mRNA isolated from cells grown in either FBS- or dFBS-supplemented MEM suggested that recovered mRNAs were functionally equivalent.

An assessment of the relative abundance of H1 and H2 expression was reversed by the addition of 10 mM 8-bromo-cGMP to the cultures. The rate that receptor concentration was lost parallels the reduction in protein interactions or reflected a more direct effect on the two independent gene transcripts, the levels of expression of H1 and H2 mRNAs were determined. Northern blot analysis of mRNA using the H1 and H2 probes indicated a small but reproducible difference in the molecular mass of the transcript (Fig. 2). The message detected by the H2-related probe corresponds to the approximately 1.5-kilobase transcript previously reported for H2 in HepG2 cells (18). When the H2 probe was used in the selection of clones from a HepG2 library, sequencing of positive clones indicated that only H2 inserts were obtained (data not shown). Based on these results, the H1 and H2 probes were used to define the level of expression of the two mRNAs.

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lated from biotin-deprived HepG2. Cells were maintained in MEM supplemented with 10% FBS from the assay blank. The resulting fluorogram was quantified by densitometry. The addition of 8-bromo-cGMP to Fig. 5. Effect of 8-bromo-cGMP on the association of ASGR mRNAs with the membrane-bound polysomes. HepG2 were maintained for 3 days in media with 10% FBS (lane A), 10% dFBS and 10 mM 8-bromo-cGMP (lane B), or 10% dFBS (lane C) before polysome isolation, as described under "Materials and Methods." Specific mRNAs associated with the membrane-bound polysomes were detected by Northern blot analysis, as described in the legend to Fig. 3. Ab., albumin.

mRNAs associated with the membrane-bound polysomes in control cells, as compared with cells grown in MEM supplemented with dFBS and cells grown in MEM plus dFBS containing 8-bromo-cGMP (Fig. 5) revealed that the amounts of ASGR mRNAs associated with membrane-bound polysomal fraction were substantially reduced in cells grown in MEM plus dFBS alone. Reprobing this transfer with an albumin-specific cDNA probe, a protein translated on membrane-bound polysomes, indicated that the reduction of recoverable ASGR mRNA was not due to a generalized dissociation of the polysomal fraction.

To compare the distribution of ASGR mRNA in the ribonucleoprotein (RNP) and polysomal fractions, a postmitochondrial supernatant prepared from cells maintained for 3 days in MEM supplemented with FBS or dFBS plus or minus cGMP was resolved on a sucrose gradient. RNA purified from each fraction of the gradients was probed with the H1 probe (Fig. 6). Almost all the ASGR-related mRNA from control and cGMP-treated cells was recovered in four sucrose density fractions (lanes 2, 3, 9, and 10) described as containing the RNP (lanes 2 and 3) and membrane-bound polysomes (lanes 9 and 10) as isolated from liver (27). The ASGR-related mRNA from cells maintained in medium supplemented with dFBS alone was confined to the lower denser sucrose RNP fractions. This distribution of ASGR mRNA was consistent with that observed in the preparation of membrane-bound polysomes (Fig. 5). Adjusting the postmitochondrial supernatant to 100 mM EDTA prior to resolution resulted in the loss of recoverable H1 mRNA within the sucrose gradient (data not shown).

**DISCUSSION**

The addition of biotin to cultures of HepG2 grown in medium supplemented with dFBS has been shown to be sufficient for the maximum expression of both H1 and H2 subunits of ASGR (5). A link between the vitamin's effect on ASGR expression and the intracellular levels of cGMP gained support from the induction of normal levels of ASGR by the addition of the cell-permeant 8-bromo-cGMP to biotin-deprived cells (8). The temporal relationship of these events (Fig. 1) is consistent with cGMP as a modulator of ASGR synthesis in confluent cultures of HepG2. Biotin and cGMP administration have been shown to increase hepatic glucokinase (28). Changes in the levels of the enzyme were shown to be the result of increased rates of transcription of the glucokinase gene (29). In contrast, when cells were grown in the absence of cGMP, no change in the steady-state concentrations of H1- or H2-specific mRNA could be detected (Fig. 5). Such an alteration in protein expression without a discernible change in mRNA concentration suggests that this regulation takes place at a posttranscriptional level.

In light of the documented dependence of H2 expression on its oligomerization with the H1 subunit (22–25), the relative abundance of both mRNA associated with the membrane-bound polysomal fraction was determined (Fig. 5). The increase in H1 and H2 mRNAs recovered associated with the polysome isolated from the membrane fraction of cells grown in MEM supplemented with dFBS containing cGMP suggests that both ASGR mRNAs can be stored in a masked or inactive form. Resolution of the postmitochondrial supernatant indicated that this inactive mRNA was associated with the RNP. Other proteins besides ASGR are regulated by controlling mRNA storage in a polysome-free, translationally repressed state that can be shifted to a polysome-associated, translationally active state (for review see Refs. 30 and 31). One of the most extensively studied models of regulated translation is the induction of ferritin by iron (14, 32, 33). A cis-acting element (the iron response element) has been identified within the 5′-nontranslated leader region of the human ferritin H-chain mRNA (33); for review see Ref. 34). As this is becoming a more commonly recognized mode of posttranscrip-
ional regulation (35–37), a cis-acting element within both the H1 and H2 mRNA might also be expected.

The selective nature of the posttranscriptional control of ASGR expression by cGMP (5) suggests the presence of a transacting factor(s) that binds to specific mRNAs, preventing the interaction with ribosomes and other components essential for protein synthesis. An example of such a factor is the protein that represses the translation of ferritin mRNA by binding to a stem-loop structure in the 5’-nontranslated leader region of its mRNA (35). Interaction of such proteins with the mouse P21 mRNA sequestered in the translationally inactive mRNP particles has been suggested by RNase T1 protection assays (37). A recent review by Scherrer (38) describes additional evidence for the involvement of RNP particles in translational control. Regulation of ASGR at a physiologic and pathologic conditions is by a common mechanism.

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REFERENCES