The Cytoplasmic Coatomer Protein COPI

A POTENTIAL TRANSLATIONAL REGULATOR

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Expression of the asialoglycoprotein receptor (ASGR) by the human hepatocellular carcinoma cell lines HepG2 and HuH-7 in response to intracellular cGMP concentrations was previously shown to be regulated at the translational level (1). Stabile transfection of COS-7 cells with deletion constructs encoding the asialoglycoprotein receptor H2b subunit localized the cGMP-responsive cis-acting element to the mRNA 5′-untranslated region. Resolution by anion exchange chromatography of an S-100 isolated from human liver resulted in the partial purification of an RNA-binding protein specific to this cis-acting element. Northwestern analysis using the 5′-untranslated region as probe indicated that a 140-kDa protein was the potential RNA-binding protein. Sequence of tryptic peptides suggested that the 140-kDa protein was the α-COP subunit of coatomer protein COPI, usually associated with trans-Golgi network membrane traffic. Immunoblot analysis confirmed the presence of α-COP in the Mono-Q fraction as well as that of a second coatomer subunit, β-COP. Antibody-induced gel retardation supershift confirmed the identification of the RNA-binding proteins as α- and β-COP. Although the RNA recognition motif appears to reside solely in α-COP, antibody-induced supershift strongly indicated that the entire coatomer complex was the trans-acting factor. Depletion of S-100 with the antibody to β-COP confirmed that the coatomer was the sole protein binding to the ASGR mRNA 5′-untranslated region in liver cytosol and responsible for inhibition of in vitro translation of the asialoglycoprotein receptor.

The asialoglycoprotein receptor (ASGR) is the hepatocellular prototype of a cell-surface lectin responsive to the differentiated state of the liver cell (2–4). In addition to clearing asialoglycoproteins from the circulation via receptor-mediated endocytosis, ASGR on hepatocytes provides a membrane-bound site for cell-cell interactions, and it has made possible the selective targeting of chemotherapeutic agents and foreign genes (5). Recently ASGR was implicated as a site of hepatitis B virus (6) and Neisseria gonorrhoeae uptake (7). With such a diverse potential, the true physiologic function of this membrane glycoprotein remains controversial.

Expression of ASGR by the human liver cell lines HepG2 and HuH-7 was shown to depend upon the presence of biotin in the culture medium (1, 8). Although usually not considered part of a signal transduction pathway, the effect of biotin was mimicked in a non-additive fashion by the second-messenger 8-bromo-cGMP. This finding suggested that biotin maintained an intracellular cGMP level via activation of the membrane-associated guanylate cyclase (1). Northern blot and polysome analysis showed that the effect of 8-bromo-cGMP addition on ASGR synthesis was at the translational level (1). Recovery of the ASGR mRNA in the ribonucleoprotein fraction during biotin deprivation suggested that intracellular levels of cGMP might play a significant role in the initiation phase of translation.

The bimodal polysome distribution of ASGR mRNA was characteristic of a class of mRNAs that were inefficiently translated (9). Current evidence suggests that mRNAs in these functionally distinct fractions differ structurally or though interactive proteins associated with their untranslated region (UTR) (10). Transfection of COS-7 cells with various deletion constructs of the cDNA encoding the ASGR H2b subunit localized the cGMP responsive cis-acting element to a 187-nucleotide fragment of the 5′-UTR (11). Inhibition of gel retardation with a nested set of RNAs limited the cognate sequence to 37 nucleotides encompassing two regions of potential secondary structure.

In the present study, a coatomer protein, COPI, comprising seven unrelated subunits was identified as the trans-acting factor bound to the 5′-UTR responsible for the translational regulation of ASGR. This finding opens the novel possibility that membrane-bound proteins when in the free state may play a different regulatory role.

EXPERIMENTAL PROCEDURES

Preparation of RNA-binding Protein—Human liver cytosol was isolated from perfused human liver stored at −80 °C. The liver was thawed and homogenized on ice in 10 mM Hepes-KOH, pH 7.8, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM dithiothreitol, and 0.1% (w/v) Sigma protease inhibitor mixture (buffer A). Homogenate was clarified for 30 min at 15,000 × g at 8 °C, and the supernatant was centrifuged for 1 h at 100,000 × g, 4 °C. The resulting supernatant was dialyzed overnight at 4 °C against 20 mM Hepes-KOH, pH 7.8, 10% glycerol, 100 mM KCl, 0.2 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, and 0.5 mM dithiothreitol (buffer D). Dialyzed supernatant clarified by centrifugation for 1 h at 100,000 × g, 4 °C was designated S-100. S-100 adjusted to 0.3 M KCl was loaded onto a 5-ml Bio-Rad Mono-Q column and washed with 0.3 M KCl in buffer D until the A280 was below 0.05. Proteins were eluted with a 0.3–0.6 M KCl linear gradient in buffer D. Two peaks containing RNA binding activity, as measured by dot blot, eluted at 0.358 and 0.385 M KCl were concentrated on Amicon Centricon Plus 30, 30-kDa cut-off spin filters. Proteins within each peak were resolved on 4–20% SDS-PAGE, and visualized by Coomassie Blue staining. Protein recovered from the SDS-PAGE was sequenced in the Laboratory for Macromolecular Analysis, Albert Einstein College of Medicine.

In Vitro Transcription and Translation—[α-32P]CTP-labeled RNA transcripts of high specific activity (>108 cpm/ug of RNA) were synthesized from a Sma-1 linearized pGem4Z plasmid containing a 187-base pair fragment of the ASGR H2b-subunit 5′-untranslated region. Promega’s Riboprobe System-SP6 was used according to the manufacturer’s directions. Bio-Rad Micro Bio-Spin 30 columns were used to...
Protein concentration of the supernatant was assayed by bicinchoninic acid precipitation according to the manufacturer’s directions. 

**Northwestern and Dot Blot**—Protein resolved on a 4–20% SDS-PAGE were transferred to nitrocellulose with 25 mM Tris, 192 mM glycine, and 20% methanol, pH 8.3 (12) using a Bio-Rad Trans-Blot SD semi-dry transfer cell at 50 mA overnight. Protein bands possessing RNA binding activity were visualized either by enhanced chemiluminescence, using horseradish peroxidase-conjugated streptavidin and Pierce Ultra substrate with biotinylated RNA, or by autoradiography with [α-32P]CTP-labeled mRNA (1–104 cpmp, 0.2–0.8 ng). For either probe, nitrocellulose from the overnight transfer or dot blot was preincubated in 12 mM Hepes-KOH, pH 7.9, 15 mM KCl, 0.2 μM dithiothreitol, partially purified yeast core RNA (0.58 g/ml), and 15% glycerol (buffer C) for 10 min at room temperature. The appropriate RNA probe was added in buffer C and continuously agitated for 20 min at room temperature. The nitrocellulose was washed three times in buffer C over a 30-min period and developed either by chemiluminescence or autoradiography (13). A Schleicher & Schuell Minifold I Microsample filtration device was used to apply Mono-Q fractions (50 μl) to nitrocellulose for dot blots and the Northwestern protocol used to determine fractions possessing RNA binding activity.

**Western Blot Analysis**—Protein fractions were resolved on either 10 or 4–20% gradient SDS-PAGE and transferred to nitrocellulose (12). The nitrocellulose was blocked for 30 min with 10% fat-free milk dissolved in TBS plus 0.05% Tween 20 (TTBS) at room temperature and constant agitation. Primary antibody was applied in fresh TTBS at 1:1000 dilution for 1 h at room temperature with constant agitation. The nitrocellulose was washed three times in TTBS over a 30-min period. Secondary horseradish peroxidase-linked antibody was added at 1:2500 dilution in fresh blocking buffer for 1 h at room temperature with constant agitation. The nitrocellulose washed three times in TTBS over a 30-min period was exposed to Pierce Ultra substrate for enhanced chemiluminescence. Anti-α-COP was kindly provided by Dr. Cordula Harter, Heidelberg University. Mouse monoclonal anti-β-COP was obtained from Sigma.

**Gel Retardation Assay**—The protocol for the gel retardation assay was adapted from that previously described by Leibold and Munro (14). Protein fractions were incubated on ice for 30 min in 10 mM Hepes-KOH, pH 7.5, 3 mM MgCl2, 40 mM KCl, 1 mM dithiothreitol, and 5% glycerol plus 1 unit of RNase inhibitor (Prime Inhibitor obtained from 5 Prime — 3 Prime, Inc., Boulder, CO)/20 μl of reaction mix. Buffer D was used to complete all reaction volumes. The RNA probe (~1–2 × 104 cpmp/reaction) was added, and the samples were incubated on ice for 30 min. Heparin (10 μg/ml final concentration) obtained from Sigma was added, and the samples were incubated on ice for an additional 10 min. Samples were loaded on a 6% low cross-link 60:1 PAGE (pre-cooled to 10 °C and pre-run for 30 min at 75 V). The loaded gel was run at 75 V, 10 °C for 3 h in 0.5× Tris-buffered borate–EDTA, after which the gel was processed for autoradiography. The specificity of the gel shift assay had been previously established by the addition of a nested set of RNA probes overlapping the putative cognate sequence within the 5′-UTR (11).

**Immunodepletion of S-100 by Anti-β COP**—Mouse monoclonal anti-β COP (25 μg) or anti-βLDL receptor (25 μg) antibodies obtained from Sigma were incubated with 50 μl of immobilized protein A/G (Pierce) in 0.5 ml of phosphate-buffered saline, pH 8.0, for 1 h with constant mixing at 4 °C. Unbound protein was removed by three successive washes with buffer D. Twenty-five μl of the antibody-protein A/G matrix was mixed with an equal volume of the S-100 fraction, and the mixture was incubated at 4 °C with constant mixing for 3 h. The immunodepleted S-100 was recovered by centrifugation at 5000 × g for 10 min at 4 °C and used immediately in translation inhibition studies. Protein concentration of the supernatant was assayed by bicinchoninic acid protein assay kit (Pierce).

**RESULTS AND DISCUSSION**

A dot blot assay in combination with a protein-specific gel retardation assay was developed to detect the presence and follow purification of the RNA-binding protein. It was assumed that the cGMP responsive trans-acting factor first detected in liver cell lines (HepG2 and HuH-7) would be present in normal human liver. Gel permeation chromatography of the human liver S-100 fraction on a Superose-6 preparative column resolved the RNA binding activity near the void volume. Calibration of the column indicated that the activity was recovered in a fraction with an apparent molecular mass of 670 kDa.

Preliminary studies indicated that the RNA-binding protein activity present in the S-100 fraction eluted from a Mono-Q column at 0.4 mM KCl. Refinement of the KCl gradient resulted in the partial resolution of two protein peaks. The first peak eluted at 0.358 mM KCl and the second peak at 0.385 mM KCl. Both peaks possessed active RNA-binding protein by the dot blot assay. Fractions with the highest apparent specific activity within each peak were pooled, concentrated by centrifugal filtration, and assessed for RNA binding activity by gel retardation. This assay was previously shown specific for this RNA fragment (11). Titration to determine the minimum amount of protein required for a positive gel shift indicated a 35- and 150-fold increase in specific RNA binding activity for pools 1 and 2, respectively, when compared with S-100. Pool 1 protein gave a shift similar to the original S-100 fraction, whereas the pool 2 protein caused a more pronounced shift (Fig. 1).

Resolution of the two pools on a 4–20% SDS-PAGE indicated that each was heterogeneous, containing at least six to nine major protein bands when visualized with Coomassie Blue stain. Northwestern analysis identified a protein within pool 2 with an apparent mass of 140 kDa as a potential RNA-binding protein (Fig. 2). This finding was consistent with our initial Northwestern analysis of the S-100 fraction. Sequence analysis of tryptic peptides isolated from the SDS-PAGE-resolved band identified the 140-kDa protein as the α subunit of the coatamer protein, COPI. Western blot analysis confirmed the identification of this protein as α-COP (Fig. 2). β-COP, a second COP1 subunit with an apparent molecular mass of 110 kDa, was also identified in this fraction (Fig. 2). Western blot also indicated the presence of both COPI subunits in the pool 1 fraction (data not shown).

The presence of both α- and β-COP in the same protein fraction strongly suggested that the observed heterogeneity on SDS-PAGE was because of the dissociation of the COPI subunits. Indeed, the SDS-PAGE banding pattern of peak 2 was consistent with the reported subunit composition of COPI, a cytosolic complex of seven unrelated subunits (15, 16). Protein elution from the Mono-Q column with a linear KCl gradient, a central step in most protocols, designed to purify COPI (15, 16), consistently yielded 600–800 μg of protein/1 g of cytosol. When compared with reported cytosolic concentrations (15, 16),2 this preparation of COPI would be on the order of 30–50% pure.

To determine whether the entire COPI complex was indeed associated with the 5′-UTR, anti-β-COP and anti-α-COP antibodies were added to the gel retardation assay in an attempt to promote a supershift. The observed enhanced retardation of the radiolabeled 5′-UTR probe by either antibody strongly suggested that the entire coatamer complex was associated with COPI RNA-binding Protein

FIG. 1. Gel retardation assay of the pool 1 and pool 2 fractions. Pool 1 (10 μg) and pool 2 (4 μg) proteins were incubated with the 187-nucleotide RNA probe (2 × 104 cpmp) before resolution on a 6% low cross-link native gel. The gel was dried and the bands localized by autoradiography.

2 G. M. Waters (Princeton University), personal communication.
that immunodepletion of the S-100 fraction. An undefined 45-kDa protein was detected with the β-COP immunodepletion strongly indicated that COPI was the sole protein in human liver cytosol specifically bound to the ASGR mRNA 5′-UTR.

An in vitro translation assay was utilized to establish a causal relationship between the presence of COPI in S-100 and ASGR translation (Fig. 5). Capped ASGR mRNA was synthesized and translated in a wheat germ system to which an immunodepleted S-100 was added (see Fig. 4). Untreated S-100 or S-100 treated with the nonspecific antibody (anti-LDL) inhibited ASGR translation by 80%. In contrast, S-100 treated with anti-β-COP inhibited translation by only 28%. Both antibody-treated S-100s exhibited an equal COPI-independent nonspecific inhibition (35%) when tested against Xenopus elongation factor 1α-capped mRNA (the positive control provided with the Ambion in vitro translation kit). These findings indicate that immunodepletion of β-COP results in a highly significant loss of specific inhibitory activity.

Formation of COPI-coated vesicles in trans-Golgi network traffic has been well established, although the exact order of recruitment and composition of potential coatomer subcomplexes associated with Golgi membranes remains controversial. From previous studies, it was evident that cells deprived of biotin or cGMP expressed higher levels of active RNA-binding protein (11). To determine whether this reflected a change in a single or multiple proteins in the S-100 fraction binding specifically to the ASGR mRNA, S-100 was immunodepleted of β-COP and associated proteins. Western blot analysis was unable to detect the presence of either α- or β-COP in the depleted S-100 (data not shown). Loss of gel shift capacity of the immunodepleted S-100 (Fig. 4) strongly suggested that COPI was the sole protein in human liver cytosol specifically bound to the ASGR mRNA 5′-UTR.

In the present study, Northwestern analysis of the SDS-PAGE-resolved, partially purified COPI coatomer suggested that the RNA (Fig. 3). Although it does not exclude the possibility that subcomplexes of COPI could associate with the 5′-UTR, as has been suggested for interaction with Golgi membranes (15), no subcomplex containing both α- and β-COP has ever been detected (17, 18).

The extent of translation was determined by trichloroacetic acid precipitation in three independent experiments and confirmed by SDS-PAGE followed by fluorography (see insert). The results are expressed as the percent of trichloroacetic acid-precipitated, radiolabeled protein in the absence of added S-100.

Subunit heterogeneity due to post-translational modification of COPI proteins forming functionally distinct coatomer complexes has also been suggested (17, 19). Dissociation/reassociation experiments have indicated that various subcomplexes were formed in vitro; however, only those containing β-COP were capable of specific binding to Golgi membranes (15). In contrast, in vivo pulse-chase and co-immunoprecipitation suggested that cytoplasmic COPI was rapidly formed from newly synthesized subunits and exists as a stable complex (18). More recently, the β′-COP subunit has been identified as a receptor for activated protein kinase C in cardiac myocytes (20). Although this demonstrates an ambifunctional role for the β′ subunit, the absence of β-COP co-localization with this kinase receptor suggested that β′-COP was not associated with the entire coatomer complex. This finding opens the possibility that individual coatomer subunits might function independently.

In the present study, Northwestern analysis of the SDS-PAGE-resolved, partially purified COPI coatomer suggested that α-COP was the most likely candidate for RNA recognition (Fig. 2). However, this result does not exclude the possibility of a coatomer accessory protein as the primary responder to intracellular cGMP. Antibody-induced supershift and immunodepletion strongly indicated that β-COP was also part of the binding complex (Figs. 3–5). Until an intermediate α/β subcom-
plex has been confirmed in vitro or in vivo (15, 17, 18) we must assume that the entire COPI complex binds to the 5′-UTR. These results suggest that COPI is the cGMP-responsive protein; however, the effects of cGMP on COPI are not known, and future experiments will be required to establish a direct link between COPI and cGMP action.

REFERENCES