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Supplemental materials and methods

Affinity purification of the anti-Cog2 Cpep antibody

The rabbit polyclonal anti-Cog2 Cpep antibody (anti-ldlCp Cpep; Podos, S.D., P. Reddy, J. Ashkenas, and M. Krieger. 1994. *J. Cell Biol.* 127:679–691) was affinity purified using a thioredoxin/human Cog2 COOH-terminal fragment (amino acids 701–738) fusion protein attached to NHS-activated Hitrap column (Amersham Pharmacia Biotech) according to the manufacturer's recommendations. The column-bound antibody was eluted by 0.1 M glycine (pH 2.0) and immediately neutralized to pH ~7.0. The fusion protein, expressed in *Escherichia coli* from the expression plasmid pCog2-Cterm, which was constructed by inserting a 730-bp ScaI/Not I fragment of p*LDLC*-1 (human Cog2/ldlCp cDNA, (Podos, S.D., P. Reddy, J. Ashkenas, and M. Krieger. 1994. *J.Cell Biol.* 127:679–691) into the pET32 expression vector (Novagen), was purified using His-Bind resin (Novagen) according to manufacture's recommendations.

Generation and affinity purification of the anti-Cog1 polyclonal antibody

The rabbit polyclonal anti-Cog1 antibody was generated using a recombinant full-length murine Cog1, His-tagged at the NH_2 terminus, as the immunogen. The plasmid pCog1N–His was constructed by ligating the 3.0-kb Not I/Not I fragment of p*LDLB*-1 (murine Cog1/ldlBp cDNA; Chatterton, J.E., D. Hirsch, J.J. Schwartz, P.E. Bickel, R.D. Rosenberg, H.F. Lodish, and M. Krieger. 1999. *Proc. Natl. Acad. Sci. USA.* 96:915–920) into pET28 (Novagen), and the recombinant protein was expressed in the BL21(DE3) host (Novagen) according to the manufacturer's recommendations. After lysis of the cells by sonication in 6 M guanidine-HCl, 300 mM NaCl, 50 mM sodium phosphate (pH 7), the protein was purified by TALON metal affinity resin chromatography (CLONTECH Laboratories, Inc.) under denaturing conditions according to the manufacturer's recommendations, except that guanidine-HCl was replaced by 8 M urea in the wash and elution buffers. An IgG fraction from the polyclonal anti-Cog1 antiserum was prepared using Hitrap–protein A chromatography (Amersham Pharmacia Biotech) according to the manufacturer's recommendations. The IgG fraction was then affinity purified by incubation with nitrocellulose membrane (Bio-Rad Laboratories) strips to which a GST–full-length Cog1 protein was bound, followed by elution with 0.1 M glycine as described above for anti-Cog2 Cpep. The GST–Cog1 fusion protein was expressed from a plasmid generated by subcloning a 3.0-kb EcoRI/EcoRI fragment from p*LDLB*-1 into pGEX5X-1 (Amersham Pharmacia Biotech). *E. coli* cells expressing this protein were lysed with buffer A (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol), and the lysates were subjected to electrophoresis and blotting onto nitrocellulose membranes. After visualization of protein bands with the dye Ponceau S, the membrane strips containing the fusion protein were isolated.

Purification of the COG complex

All procedures were performed at 4°C. Solutions were buffered with 25 mM TrisCl, pH 8.0, 1 mM DTT (TD) and contained the indicated amounts of KCl (e.g., TD + 1 M KCl) and glycerol, unless otherwise noted. Bovine brain cytosol (48 g protein) was ammonium sulfate precipitated as described previously (Waters, M.G., D.O. Clary, and J.E. Rothman. 1992. *J. Cell Biol.* 118:1015–1026). The 12 g of precipitated protein was resuspended in 510 ml of TD + 50 mM KCl. The conductivity of this solution was adjusted to that of TD + 1 M KCl by the addition of TD + 3 M KCl, and then the volume was adjusted to 1,200 ml with TD + 1 M KCl. This sample was incubated with a 400-ml bed volume of butyl-Sepharose preequilibrated with TD + 1 M KCl. After assembly into a column, the resin was washed with TD + 1 M KCl at 2 ml/min until the UV absorbance of the eluate was near baseline. Bound COG complex was eluted with TD + 10 mM KCl at a flow rate of 2 ml/min. The fractions containing the bulk of the eluted protein were pooled (660 ml), concentrated to 280 ml using an Amicon pressure cell with a 30-kD cutoff membrane, and dialyzed for 2 h against 4 liters of TD + 100 mM KCl. After the concentration of KCl in the TD buffer was adjusted to 160 mM KCl, the sample was chromatographed in four 70-ml batches at 1 ml/min on a MonoQ HR10/10 column, using a 30-ml TD + 160 mM KCl wash, and a 35-ml 160–300 mM KCl elution gradient. Fractions containing Cog5 immunoreactivity (centered around 220 mM KCl) were pooled and dialyzed overnight against 500 ml of 25 mM Hepes-KOH, pH 7.0, 100 mM KCl, 10% glycerol. The dialyzed fractions contained 48 mg of protein. An aliquot containing 44 mg was adjusted to include 5 mM potassium phosphate (KP_i) by addition of 1 M KP_i (pH 7.0) and loaded at 0.75 ml/min onto a 5-ml ceramic hydroxyapatite column (Bio-Rad Laboratories) equilibrated in 25 mM Hepes-KOH, pH 7.0, 100 mM KCl, 10% glycerol, 5 mM KP_i. This column was washed with 20 ml of equilibration buffer and bound COG complex was eluted with a 30-ml 5–30 mM KP_i gradient in 25 mM Hepes-KOH, pH 7.0, 100 mM KCl, 10% glycerol. Fractions were immediately dialyzed against TD + 100 mM KCl containing 10% glycerol in a microdialyzer

(GIBCO BRL) and the COG complex–containing fractions were identified by immunoblotting. The second peak of Cog5 immunoreactivity (Fig. 1 A, peak 2) was concentrated to 350 μl using a centrifugal concentrator (Millipore) and loaded at 0.25 ml/min onto a Superose 6 HR10/30 sizing column equilibrated in TD + 100 mM KCl, 10% glycerol. COG complex immunoreactivity was highest in the eluate between 9.6 and 10.9 ml and these fractions were pooled and loaded at 0.15 ml/min onto a 240-μl SMART MiniQ (Amersham Pharmacia Biotech) column equilibrated with TD + 100 mM KCl, 10% glycerol. The column was washed with 1.5 ml of TD + 170 mM KCl, 10% glycerol and eluted with a 1.25 ml, 170–270 mM KCl gradient in TD + 10% glycerol. An estimated 70 μg of COG complex eluted at ∼220 mM KCl.

Analytical gel filtration of the two different Cog5-containing complexes (Fig. 1 B, peak 1 and 2) was performed on a 2.4-ml SMART Superose 6 3.2/30 column (Amersham Pharmacia Biotech) at a flow rate of 50 μl/min in TD + 100 KCl, 10% glycerol. Fractions of 80 μl were collected starting at 0.88 ml after injection.

Deep etch EM of purified COG molecules

To visualize single COG complex particles, ∼20 μg of purified COG complex (Fig. 2 A, fraction 18) was visualized on mica by quick freeze/deep etch/rotary shadowing microscopy as previously described (Heuser, J.E. 1983. *J. Mol. Biol.* 169:155–195). In brief, this involved mixing two drops of a suspension of finely ground mica flakes with 0.5 ml of an ∼10 μg/ml solution of highly purified COG complex and allowing the protein to adsorb for 30 s. For this adsorption step, both the mica and the protein were suspended in an intracellular buffer we term "KHMgE" (70 mM KCl, 30 mM Hepes, pH 7.2, 5 mM $MgCl_2$, and 3mM EGTA). When glutaraldehyde fixation was required, the 10 μg/ml COG solution was mixed with a small volume of 7% glutaraldehyde, enough to bring it to 50 mM of the fixative, and incubated for 30 s before mixing it with a suspension of mica flakes, as usual. The only difference was that in this case, the mica had to be pretreated with 1 mM low molecular weight polylysine (mol wt 7,000 D) in 0.1 M KCl and washed extensively in KHMgE before use, or else no protein would stick to it (because fixed proteins have such a low isoelectric point; Heuser, J. 1989. *J. Electron Microsc. Tech.* 13:244–263). In all cases, the mica flakes were pelleted by gentle centrifugation after the 30 s of protein adsorption, immediately washed twice with KHMgE, and then layered as a thick, but not dry, slurry onto a thin slice of KHMgE-soaked and aldehyde-prefixed lung (for support during freezing). Quick freezing was accomplished by smashing the mica slurries onto a copper block cooled to ∼10° Kelvin with liquid helium.

The frozen slurry of mica flakes was then freeze fractured in a standard Balzer's freeze etching unit and immediately deep etched for 4 min at −100°C. It was then rotary replicated with ∼2 nm of platinum applied from an angle of 11° above the horizontal and backed with an ∼10 nm film of pure carbon. Replicas were separated from the mica by overnight floatation on a solution of concentrated hydrofluoric acid and finally washed several times in water, before being picked up on 75-mesh formvar-coated microscope grids. Replicas were viewed in a JEOL transmission electron microscope operated at 100 kV, and stereo images were obtained using ±10° of tilt with a eucentric side entry goniometer stage. Final anaglyph 3-D images were generated by making digital copies of the standard 3.25 × 4 inch EM negatives that represented the stereo pairs, using a 6-M pixel Kodak professional DCS 560 camera (Heuser, J. 2000. *Traffic.* 1: 614–621), and overlaying the resultant digital tiff files as separate colors (left, pure red; right, a mix of green and blue), using for the final overlay the screen command in the Layers control panel of Adobe Photoshop® (Heuser, J. 2000. *Traffic.* 1:35–37).

Immunoprecipitation of the COG complex from rat liver cytosol and a partially purified bovine brain COG complex preparation

Rat liver was homogenized in 0.1 M KP_i, pH 6.8, 5 mM $MgCl_2$, 0.5 M sucrose, 1 mM DTT using a Polytron homogenizer (Brinkman Instrument), and then clarified by centrifugation (∼12,000 *g*, 60 min). The resultant supernatant was subjected to further centrifugation at ∼20,000 *g* for 120 min to generate cytosol used for anti-Cog2 immunoprecipitations. Bovine brain COG complex purified through the MonoQ step was used for anti-Cog1 immunoprecipitations.

For immunoprecipitation with monoclonal anti-Cog1 antibody, 5 μg of antibody was added to 50 μl of partially purified bovine brain COG complex. For immunoprecipitation with affinity-purified anti-Cog2 antibody, 5 μg of antibody with or without antigenic peptide (see below) was added to 400 μg of rat liver cytosol. The samples were adjusted to a total of 500 μl with PBS with (for anti-Cog1) or without (for anti-Cog2) 5% (wt/vol) nonfat dried milk and incubated overnight at 4°C. Subsequently, 10 μl of a 50% (vol/vol) slurry of protein A–Sepharose CL-4B in PBS was added, and the mixture was gently swirled for 2 h at room temperature. The beads were then sedimented by brief centrifugation in a microfuge and washed four times with 500 μl PBS with (for anti-Cog1) or without (for anti-Cog2) 5% (wt/vol) nonfat dried milk and once with 500 μl of PBS. After aspiration of the excess liquid with a 25 gauge needle, the beads were boiled in SDS sample buffer for 5 min to elute the bound protein, and the samples were analyzed by immunoblotting. For anti-Cog2 antigenic peptide competition, the anti-Cog2 antibody was preincubated for 1 h at room temperature with 10 μg of the COOH-terminal antigenic peptide (Cpep; Podos, S.D., P. Reddy, J. Ashkenas, and M. Krieger. 1994. *J. Cell Biol.* 127:679–691) in 100 μl of PBS before addition to the cytosol.

Preparation of the pHM6-Cog7 expression plasmid

Full-length Cog7 cDNA was obtained by PCR from Image Clone 3163316 (Incyte Genomics), with the 5′ primer 5′-GCGTTGGCACTAAGCTTCATGGACTTCTCCAAGTTCCTG-3′ and the 3′ primer 5′-GCCTCGGTCGGTACCG-TAATTCACACTCCGCATGGTG-3′. The resulting PCR product was cloned into the pHM6 vector (Roche) with the restriction enzymes HindIII and KpnI, resulting in the plasmid pHM6-Cog7, in which an NH_2-terminal HA epitope tag and a COOH-terminal His_6 tag are added to the coding region of Cog7.

Growth of CHO and HeLa cells

The CHO, ldlB, and ldlC cells were grown in medium A (Ham's F12 containing penicillin, 50 U/ml, streptomycin, 50 μg/ml, and glutamine, 2 mM) supplemented with 5% (vol/vol) FBS (medium B). ldlB[*COG1*] and ldlC[*COG2*] cells were maintained in medium A supplemented with 3% newborn calf lipoprotein-deficient serum, 250 μM mevalonate, 2.5 μg protein/ml LDL, and 40 μM compactin (medium C) to select for the expression of LDL receptors (Goldstein, J.L., J.A. Helgeson, and M.S. Brown. 1979. *J. Biol. Chem.* 254:5403–5409) and thus the expression of the transgenes encoding either Cog1 or Cog2 (Chatterton, J.E., D. Hirsch, J.J. Schwartz, P.E. Bickel, R.D. Rosenberg, H.F. Lodish, and M. Krieger. 1999. *Proc. Natl. Acad. Sci. USA.* 96:915–920; Podos, S.D., P. Reddy, J. Ashkenas, and M. Krieger. 1994. *J. Cell Biol.* 127:679–691).

For immunofluorescence microscopy, CHO cells were plated on day zero on 18-mm coverslips in 12-well dishes (20,000 cells/well) and processed for microscopy on day two. For immunoblotting, cells were harvested by trypsinization, washed once with growth medium and once with PBS, and suspended in 250 μl of PBS. Lysates were immunoblotted after boiling for 5 min in the presence of SDS sample buffer. For electron microscopy, cells were plated on day zero in 15 ml of growth medium in 100-mm dishes (500,000 cells/dish) and fixed and embedded on day three.

For immunofluorescence, HeLa cells were seeded onto 22-mm glass coverslips in 100-mm dishes at a concentration such that the cells were ∼50% confluent after 20 h of growth. The cells were grown in MEM supplemented with 1% 100× nonessential amino acids (GIBCO BRL), 50 U/ml penicillin G, 50 μg/ml streptomycin, 2.8 mM L-glutamine and 10% (vol/vol) FBS (GIBCO BRL). The resulting cultures were transfected with the pHM6-Cog7 plasmid using the calcium phosphate method (Ausubel, F.M., R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, and K. Struhl. 1995. Short Protocols in Molecular Biology. John Wiley & Sons, Inc., New York. 910 pp.). The cells were grown for an additional 36 h before immunofluorescence microscopy.