Role of the Chaperonin CCT/TRiC Complex in G Protein βγ-Dimer Assembly*

Christopher A. Wells, Jane Dingus, and John D. Hildebrandt From the Department of Pharmacology, Medical University of South Carolina, Charleston, South Carolina 29425

Received for publication, March 14, 2006, and in revised form, May 10, 2006 Published, JBC Papers in Press, May 15, 2006, DOI 10.1074/jbc.M602409200

The human genome codes for nearly 700 G protein-coupled receptors (1, 2), which signal through heterotrimeric G proteins and mediate the effects of a vast array of hormones, neurotransmitters, sensory signals, and drugs (3, 4). G protein-coupled receptors transmit signals to downstream effectors by catalytically activating target G proteins (5, 6). Theoretically, at least, the G protein targets of the G protein-coupled receptors are as diverse as the receptors through their combinatorial formation from 16 Gα, 5 Gβ, and 12 Gγ gene products (7). Signaling specificity of heterotrimers is affected both by the biochemistry of their protein-protein interactions and by cellular targeting mechanisms that direct placement of receptors, G proteins, and second messenger targets (7, 8). An important but poorly understood process that affects all aspects of the signaling specificity of the heterotrimeric G proteins is the synthesis and assembly of G protein heterotrimers.

Gβγ dimer formation is an early event in G protein assembly (9, 10). Biochemically, the Gβγ complex is a very stable dimer (11) that reversibly interacts with the Gα subunit, dependent on the associated guanine nucleotide (5). Given the apparent stability of the Gβγ dimer, its assembly is thought of as an all-or-none event; however, recent work indicates that the efficiency of dimer assembly in vitro is related to isoform-specific interactions of Gβ (12). Gβ is a member of a large family of WD repeat proteins (13) that contain 4–16 repeats of a core of ~40 amino acid. Gβ has seven repeats that form a closed, toroid-shaped, seven-bladed, β-propeller structure (14, 15). Folding of Gβ into this structure requires Gγ (16) and the participation of at least one unidentified components of in vitro translation mixtures (17, 18). One candidate for mediating Gβ folding and Gβγ dimer formation is the chaperonin CCT/TRiC complex (18–20).

65–80% of newly translated proteins fold spontaneously. In eukaryotes, at least three systems of molecular chaperones help fold the other 20–35% of cellular proteins: the heat shock proteins Hsp40 and Hsp70, the Hsp90 system, and the chaperonins. The GroEL-GroES complex, in which GroEL is formed by two rings of seven identical 57-kDa monomers that nonspecifically recognize proteins with exposed hydrophobic surfaces. The eukaryotic Group II chaperonins are called CCT (chaperonin containing TCP-1) or TRiC (TCP-1 Ring complex); TCP-1 is a tailless complex polypeptide-1 (22). Eukaryotic CCT/TRiC is considerably more complex than GroEL, with eight different subunits that share 30% sequence identity, and it recognizes specific but poorly defined sequences in target proteins (19, 23, 24). CCT/TRiC was originally characterized in the folding of actin and tubulin, but is now recognized to be involved in the folding of many different proteins (19, 21, 22), a subset of which includes some WD repeat proteins (19, 25), although it is not involved in the folding of all WD repeat proteins (26).

Isolated CCT/TRiC subunits were identified as interacting partners of the yeast Gβ homolog Ste4 in a high throughput screen of yeast genome coding regions (27), but this did not extend to functional characterization of these interactions or to association with the CCT/TRiC complex. More recently, small interfering RNA to TCP-1α was shown to decrease Gβγ levels in cells (20), indicating the possibility that some cellular component involved in Gβγ dimer formation is dependent on the
Gβγ Assembly on CCT/TRiC

function of CCT/TRiC, and this was proposed as a site of regulation of Gβγ dimer formation. Despite these observations, there is no direct evidence that CCT/TRiC does or even could participate either in folding of Gβ or in Gβγ dimer formation. There is not even any information about whether eukaryotic Gβ or Gβγ interacts with CCT/TRiC. Here, we have studied the mechanism of Gβγ dimer formation in rabbit reticulocyte lysate and describe the role of CCT/TRiC both in Gβ folding and in Gβγ dimer formation.

MATERIALS AND METHODS

Vectors—All Gβ and FLAG-Gγ constructs were as described previously (12). All FLAG-Gβ constructs were obtained from the Guthrie cDNA Resource Center (available at www.cdna.org).

In Vitro Translation and Transcription—In vitro translation of subunits was carried out using the TnT® Quick-Coupled Transcription/Translation System (manual no. TM045, Promega Corp.) as described previously (12). Any additions to the translation mixture were added before DNA and lysate.

Gβγ Dimerization Assay by Immunoprecipitation—Quantitation of synthesized protein and the dimerization reaction were done as described previously (12).

Immunoprecipitation of Synthesized Subunits—Immunoprecipitation of the FLAG-tagged subunits from translations and/or dimerizations was carried out using agarose-conjugated anti-FLAG antibody (catalog no. A-2220, Sigma) with 5–20 μl of beads/sample. Beads were washed twice with 50 mM Tris (pH 7.4), 100 mM NaCl (Tris-buffered saline (TBS)2), 1 mg/ml bovine serum albumin, and 0.1% C12E10 (polyoxyethylene 10-lauryl ether) (TBSBC). The beads were blocked against non-specific binding by incubation for 1 h at room temperature in 10 volumes of TBSBC with 5% reticulocyte lysate and then washed twice with 50 mM Tris (pH 7.4), 100 mM NaCl, and 0.1% C12E10 (TBSC). For immunoprecipitation, the sample was diluted 10−20-fold with TBSC; 5–20 μl of beads were added; and the samples were incubated for 1 h at room temperature. The samples were centrifuged, and the beads were resuspended in TBSC and transferred to a new tube to reduce nonspecific binding of Gβ. The samples were washed a second time. Electrophoresis sample buffer was added to the beads to elute the bound proteins.

Electrophoresis—Electrophoresis was carried out by the Laemmli procedure using Criterion Tris-HCl precast gels (Bio-Rad). Native (nondenaturing) gel electrophoresis was carried out following a procedure modified from that of Hansen et al. (28) also using Criterion gels, but SDS and β-mercaptoethanol were omitted from both sample and electrophoresis buffers. The gels were run at 4 °C at 0.4 A with an initial voltage of 140 V for 20–30 min and then 180–200 V for the remainder of the run. For autoradiography, the gel was washed twice for 15 min with 50% methanol and 10% acetic acid and then fixed for 5 min with 7% acetic acid, 7% methanol, and 1% glycerol before drying. Dried gels were exposed to film overnight at −80 °C or to a phosphor storage screen for 3–5 days at room temperature. The screen was imaged and analyzed using a GE Healthcare Storm instrument and ImageQuant software. Molecular mass standards for non-denaturing gels were from Amersham Biosciences (high molecular mass calibration kit) and included thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), lactate dehydrogenase (140 kDa), and bovine serum albumin (67 kDa).

Immunoblotting—Immunoblotting of gels was performed by transferring the gels to nitrocellulose using either a Bio-Rad Trans-Blot or Trans-Blot SD semidy transfer apparatus (29). The antibodies used were the following: rabbit anti-Gβγ antibody BC1 (30); mouse monoclonal anti-FLAG (Sigma); rat monoclonal anti-TCP-1α (Calbiochem); and goat anti-TCP-1α/β/γ/δ/ε/θ/η/ζ (Santa Cruz Biotechnology, Inc.). After transfer, the nitrocellulose was blocked with 20 mM Tris (pH 7.5), 150 mM NaCl, and 0.5% Tween 20 (TBST) with 5% nonfat dry milk and 1% bovine serum albumin, and the primary and secondary antibodies were diluted in TBST with 0.5% nonfat dry milk and 0.1% bovine serum. The primary antibody was incubated for 1–3 h and washed extensively, and then the secondary antibody was incubated for 1 h. Blots were visualized using chemiluminescence (SuperSignal West Femto, Pierce). For simultaneous visualization of two antibodies, transfers were blocked with Odyssey blocking buffer (LI-COR Biosciences) and incubated with rabbit polyclonal anti-FLAG and goat anti-TCP-1α antibodies diluted in Odyssey blocking buffer (1:1 with TBST containing 0.2% Tween 20). IRDye 700DX-conjugated donkey anti-rabbit and IRDye 800-conjugated donkey anti-goat secondary antibodies (Rockland Immunochemicals, Inc.) were diluted in Odyssey blocking buffer (1:1 with TBST containing 0.01% SDS). Immunoblots were visualized using an Odyssey infrared imaging system (LI-COR Biosciences).

Proteomics Analysis of Samples Recovered after SDS-PAGE—FLAG-tagged Gβ1 was expressed in vitro as described previously (16) and immunoprecipitated with anti-FLAG beads. Immunoprecipitated protein was eluted from the beads with SDS sample buffer without β-mercaptoethanol and was run on 4–20% Criterion gels. Gels were stained with Coomassie Blue and destained with 10% acetic acid. Bands were excised with a scalpel and further processed in a PerkinElmer Life Sciences MultiPROBE II HT EX liquid-handling robot equipped with a Millipore Montage In-Gel Digest manifold. Gel slices were sequentially equilibrated with 25 mM ammonium bicarbonate and 5% acetonitrile, then with 25 mM ammonium bicarbonate and 50% acetonitrile, and finally with 100% acetonitrile. Protein was digested by reconstituting dehydrated gel slices with 15 μl of 10 μg/ml trypsin in 25 mM ammonium bicarbonate and incubating for 3 h at 37 °C. Recovered peptides were analyzed by MALDI-TOF/TOF on an Applied Biosystems 4700 proteomics analyzer. Mass spectra were generated from 3000–5000 laser shots, and the 10 most intense protein peaks were sequenced by tandem mass spectrometry (MS/MS). The lower and upper limits of mass detection were 1000 and 3000 Da, respectively. Data were analyzed with an ABI GPS Explorer 3.5 running Mas-

2The abbreviations used are: TBS, Tris-buffered saline; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; MS/MS, tandem mass spectrometry; MS, mass spectrometer; ATPγS, adenosine 5′-O-(thiotriphosphate); ADPβS, adenosine 5′-O-2-(thiodiophosphate); PhLP, phosducin-like protein.
Analysis of Gβ and Gγ Subunits on Nondenaturing Gels—

The inference from our previous work (12) and from the data in Fig. 1, as well as from analogous experiments with mutants of conserved Asp residues in Gβ (18), was that other constituents of the reticulocyte lysate interact with Gβ isoforms and are involved in Gβ subunit dimerization with Gγ. Nondenaturing (native) gel analysis was used to identify constituents of the reticulocyte lysate that interact with either Gβ or Gγ. Interpretation of results with nondenaturing gels is more complex than with SDS-polyacrylamide gels because protein migration is determined by multiple properties, including molecular mass, pl, and shape. However, native gels preserve many interactions that would be disrupted with SDS and/or β-mercaptoethanol, and so migration is also affected by formation of protein complexes. Taking all of these factors into account, globular proteins (or complexes) of similar pl will still travel on nondenaturing gels according to their molecular mass (or log molecular mass). This was (approximately) true, for example, for standards used in the characterizing the mobility of Gβ, Gγ, and Gβγ dimers (Fig. 2), where we used a set of globular proteins with pl values of 5.4–6.8. The Gβγ dimer deviates somewhat, but not drastically, from being a globular protein (14), and it has a pl dominated by the Gβ component predicted to be between 5.6 and 6.0 for the different isoforms. Thus, our expectation was that the dimer, and perhaps the folded Gβ protein alone, would travel with an apparent molecular mass on nondenaturing gels only slightly greater than its predicted 45 kDa. On the other hand, Gγ alone is small (8 kDa) and of heterogeneous pl among its isoforms and appears to have defined structure based primarily upon its association with Gβ (14). Thus, the behavior of Gγ on nondenaturing gels was less predictable.

When in vitro translated, 35S-labeled Gγ isoforms were analyzed on native gels (Fig. 2A), they did not travel with similar mobility, as they would on SDS-polyacrylamide gels. In fact, they appeared to segregate primarily according to their pl, as shown in the accompanying graph (Fig. 2B). Those Gγ subunits with a pl near 7, i.e. Gγ2, Gγ1, Gγ4, Gγ6, Gγ7, and Gγ10 migrated in the middle of the gel. Those with a pl <7, i.e. Gγ1, Gγ13, and Gγ13, migrated more rapidly on the gel. And those with a pl >7, i.e. Gγ5, Gγ7, and Gγ12, either did not enter the gel (i.e. Gγ11) or migrated near the top. There were also two nonspecific bands that were present in all of the lanes, including the pcDNA control lane (Fig. 2A, arrows). For some Gγ isoforms, for example, Gγ10, Gγ6, and Gγ11, two bands were seen. Gγ subunits have post-translational modifications, including by farnesylation or geranylgeranylation at the C terminus and by N-terminal processing that can include removal of methionine and/or acetylation of the N terminus. Variable post-translational modification at these sites, as we have described recently for brain Gγ subunits (32), may affect the hydrophobicity and/or shape of the Gγ subunits such that their migration on nondenaturing gels is affected.

The behavior of Gβ isoforms on native gels (Fig. 2C) was quite different from that of the Gγ isoforms (Fig. 2A). For all the in vitro translated Gβ isoforms, 35S-labeled protein accumulated in two primary locations (Fig. 2C): at the top of the native gel, presumably as denatured, aggregated protein of a size too large to enter the gel, and as a sharp, well delineated band at...
Note that the position of TCP-1 positions of molecular mass standards (in kilodaltons) are shown on the left. 

BC1 is anti-rabbit. (The anti-rat secondary antibody used in the TCP-1 co-immunoprecipitation with Gβ3 at 2:3; lane 4, Gβ3, and FLAG-Gγ3 was expressed in the reticulocyte lysate and labeled with [35S]methionine. Samples were run on a nondenaturing 4 – 15% gradient gel, and the gel was dried and autoradiographed. Band a, Gβ of high apparent molecular mass; band b, nonspecific band present in all samples; band c, band seen with Gβ1, Gβ2, Gβ3, and Gβ4; band d, nonspecific band present all samples; band e, band specific to Gβ1, band f, band specific to Gβ3. The gray arrow indicates the top of the gel. 

The apparent size of the interacting protein is >669 kDa (band a). This band is specific to translation of Gβ and was not found in the pcDNA control or after translation of Gγ (Fig. 2C). There was also some labeled material near the 67-kDa marker that was diffuse for Gβ1, Gβ2, Gβ3, and Gβ4; sharper for Gβ3 (band f); and prominent but diffuse for Gβ3L (band e). This material migrated at a molecular mass slightly larger than expected for Gβ3 subunits, perhaps representing incompletely folded protein. There were also two nonspecific bands (bands b and d), also seen with Gγ (Fig. 2A), and a band of variable intensity at ~140 kDa (band c), which might be a dimer of the material at ~60–70 kDa or Gβ complexed with other specific proteins. Most interesting in these samples are the bands at >669 kDa. Because Gβ was the only significantly labeled protein on this gel (see Fig. 1A, for example), the best explanation for band a is that it is Gβ migrating at a very high molecular mass in association with a multihelical protein. The fact that this is a sharp band supports this idea because denatured and/or aggregated protein either would not enter the gel (as seen at the top) or would have heterogeneous mobility and produce a protein smear, as would incompletely or randomly folded protein.

Characterization of the Interaction of Gβ with the Chaperozin CCT/TRiC Complex—The apparent size of the interacting partner on native gels and previous reports that some WD repeat proteins interact with or are folded by CCT/TRiC (19, 25, 27) suggested that one candidate for the binding partner affecting the mobility of Gβ3 subunits on native gels is the chaperonin CCT/TRiC complex. The intact CCT/TRiC protein, with an approximate molecular mass of ~800 kDa, migrates very slowly on a nondenaturing gel (28, 33). This is shown in Fig. 3A, where samples of the reticulocyte lysate with or without expressed proteins (FLAG-Gβ3 and FLAG-Gγ3) were run on a native gel and immunoblotted with an antibody specific for the α-subunit of CCT/TRiC (TCP-1α). All these samples contained a high molecular mass band recognized by anti-TCP-1α antibody at a high molecular mass similar to that seen in the Gβ3 subunit lanes (Fig. 2). Thus, Gβ3 synthesized in the reticulocyte lysate migrates on native gels in a region also containing a high molecular mass complex of the CCT/TRiC subunits. To more definitively evaluate whether Gβ and CCT/TRiC co-migrate on nondenaturing gels, we stained the immunoblot with antibodies tagged with infrared dyes that are amenable to double staining of immunoblots (Fig. 4). FLAG-Gβ1, FLAG-Gβ2, or the pcDNA control was translated in the reticulocyte lysate and then separated on either nondenaturing (Fig. 4A) or denaturing (SDS) (Fig. 4B) gels. Proteins transferred to nitrocellulose were

**FIGURE 2. Separation of Gγ and Gβ subunits on native (nondenaturing) gels.** A. native (nondenaturing) gel of Gγ subunits. Gγ subunits were expressed in the reticulocyte lysate and labeled with [35S]methionine. Samples were run on a nondenaturing 4 – 15% gradient gel, and the gel was dried and autoradiographed. Arrowheads indicate positions of Gγ; arrows indicate nonspecific bands present in all samples. B, pl value for Gγ. The pl values for the Gγ subunits were predicted using the Compute pl/Mw tool available on the ExPASy proteomics server (available at us.expasy.org/tools/pi_tool.html). C, native (nondenaturing) gel analysis of Gβ subunits. Gβ subunits or FLAG-Gγ were expressed in the reticulocyte lysate and labeled with [35S]methionine. Samples were run on a nondenaturing 4 – 15% gradient gel, and the gel was dried and autoradiographed. Band a, Gβ of high apparent molecular mass; band b, nonspecific band present in all samples; band c, band seen with Gβ1, Gβ2, Gβ3, and Gβ4; band d, nonspecific band present all samples; band e, band specific to Gβ3; band f, band specific to Gβ3. The gray arrow indicates the top of the gel.

**FIGURE 3. Behavior of CCT/TRiC on native (nondenaturing) gels and its co-immunoprecipitation with Gβ.** A, immunoblot of CCT/TRiC on a nondenaturing gel. Gβ3, and FLAG-Gγ3 were synthesized with unlabeled methionine and combined to allow dimer formation. Samples were run on an 8 – 16% nondenaturing gel, transferred, and immunoblotted (IB) with antibody to the CCT/TRiC subunit TCP-1α. Lane 1, Gβ3 alone; lane 2, FLAG-Gγ3; lane 3, Gβ3, and FLAG-Gγ3 at 1:1; lane 4, Gβ3, and FLAG-Gγ3 at 2:1; lane 5, Gβ3, and FLAG-Gγ3 at 1:2. Lane 6, GST-pcDNA3. The approximate positions of molecular mass standards (in kilodaltons) are shown on the left. Note that the position of TCP-1α on the gel, traveling slightly slower than the 669-kDa standard, is similar to the position of the Gβ3 band labeled a in Fig. 2C. B, Gβ3 co-immunoprecipitates with CCT/TRiC. Unlabeled FLAG-Gβ3 and unlabeled FLAG-Gγ3 were separately expressed and immunoprecipitated (IP) using anti-FLAG beads. Duplicate samples were separated by SDS-PAGE on an 8 – 16% gel, transferred, and immunoblotted with rat monoclonal anti-TCP-1α antibody (left) or rabbit anti-Gβ3 subunit antibody BC1 (right). Lanes 1, FLAG-Gβ3; lanes 2, FLAG-Gγ3; lanes 3, pcDNA3. The approximate positions of molecular mass standards (in kilodaltons) are indicated on the right. The secondary antibody for TCP-1α is anti-rabbit, whereas the secondary antibody for BC1 is anti-rabbit. (The anti-rabbit secondary antibody used in the TCP-1α immunoblot recognized mouse heavy and light chains (indicated with gray arrows on the left) that co-eluted from the anti-FLAG beads (containing a mouse monoclonal antibody).) Note that TCP-1α co-immunoprecipitated only with FLAG-Gβ3 (lanes 1), but not with FLAG-Gγ3 (lanes 2) or with anti-FLAG beads from a mock translation (lanes 3).
FLAG (donkey anti-rabbit IgG) emitting at 700 nm and colored bodies were tagged with infrared-emitting labels: secondary antibody to TCP-1 protein (goat) and as the simultaneous signal, with anti-FLAG antibody (rabbit) and anti-TCP-1 antibody (goat) antibodies. Secondary antibodies were tagged with infrared-emitting labels: secondary antibody to FLAG (donkey anti-rabbit IgG) emitting at 700 nm and colored red and secondary antibody to TCP-1α (donkey anti-goat IgG) emitting at 800 nm and colored green. Immunoblots were scanned in an Odyssey infrared imager (LI-COR). In each case, the identical immunoblot is shown with its red-emitting signal (anti-TCP-1 antibody) and its green-emitting signal (anti-FLAG-1 antibody) and as the simultaneous signal, with yellow indicating co-localization of the two signals. The approximate positions of molecular mass markers (in kilodaltons) are shown on the left. Note that Gβ and TCP-1α traveled at their subunit molecular masses in A, but co-migrated at high apparent molecular mass in A, an apparent molecular mass that is compatible with that of the intact CCT/TRiC complex.

blotted simultaneously with anti-TCP-1α and anti-FLAG antibodies and visualized with secondary antibodies tagged with different infrared-emitting dyes (green for TCP-1α and red for FLAG in Fig. 4). On SDS-polyacrylamide gels, Gβ and TCP-1α traveled independently of one another and at their expected monomer molecular masses (Fig. 4B). On nondenaturing gels, however, Gβ and TCP-1α traveled at a high molecular mass that could be co-localized as a merged signal of the two tagged antibodies. These data demonstrate that Gβ and TCP-1α co-migrate on nondenaturing gels, suggesting their association in a complex.

To determine whether Gβ and the CCT/TRiC subunits are actually physically associated, we evaluated whether the subunits co-immunoprecipitate. Unlabeled FLAG-Gβ, FLAG-Gγ, or the pcDNA control was expressed in lysate and subjected to immunoprecipitation with anti-FLAG beads (Fig. 3B). Only the FLAG-Gβ immunoprecipitates contained TCP-1α immunoreactivity. Notably, the FLAG-Gγ and pcDNA control samples contained only the heavy and light chains from the anti-FLAG antibody and nothing in the position of TCP-1α or Gβ. Thus, FLAG-Gβ, but not FLAG-Gγ, binds to TCP-1α. To more generally evaluate the specificity of association of CCT/TRiC with Gβ and to confirm that the immunoreactivity observed in Fig. 3 was in fact TCP-1α, we stained SDS gels of immunoprecipitates with Coomassie Blue (Fig. 5A). In comparing control (pcDNA3) and FLAG-Gβ1 samples, we observed several protein bands between 50 and 60 kDa associated with the FLAG-Gβ1 sample (Fig. 5B). The specificity of this complex of proteins for association with FLAG-Gβ was striking given that reticulocyte lysate is essentially intact cytosol containing a large amount of hemoglobin (Fig. 5C). To identify the constituents of this complex, we used mass spectrometric (MS) peptide map-
TABLE 1

| Band | Rank | No. related matches | ID | Species | Description | Mass (kDa) | Peptide count | MS/MS-seq. peptides | Total protein score | Protein score C.I. |
|------|------|---------------------|----|---------|-------------|------------|--------------|-------------------|--------------------|--------------------|
| 1    | 1    | 10 (10)             | gi 347839 | Mouse   | Matrin, novel TCP-1-related protein | 60,450     | 22           | 6                | 323.0              | 100.0              |
| 2    | 1    | 10 (10)             | gi 135536 | Hamster | TCP-1α      | 60,301     | 15           | 6                | 446.0              | 100.0              |
| 3    | 1    | 10 (10)             | gi 34867525 | Rat   | Predicted CCT/TRiC8 | 59,550     | 15           | 7                | 448.0              | 100.0              |
| 4    | 1    | 9 (9)               | gi 64017  | Mouse   | Chaperonin | 58,085     | 13           | 5                | 349.0              | 100.0              |
| 5    | 1    | 1 (0)               | gi 782065 | Mouse   | Igγ, heavy chain | 17,508     | 0            | 5                | 59.7               | 89.1               |
| 6    | 1    | 4 (4)               | gi 5440073 | Rat   | TCP-1β      | 57,422     | 14           | 3                | 158.0              | 100.0              |
| 7    | 5    | 6 (0)               | gi 110077 | Mouse   | Igγ, chain C | 17,305     | 5            | 0                | 52.2               | 38.6               |

* Bands were recovered from the Coomassie Blue-stained gel shown in Fig. 5.

* The NCBI accession number is for the protein indicated.

* The species is for the identified protein.

* Listed is the predicted molecular mass of the identified protein.

* The number of mass peaks in the MS record within 1 Da of the predicted mass of a trypsin fragment from the identified protein is given.

* The values indicate the number of the 10 most intense mass peaks that were sequenced by MS/MS and that generated a sequence compatible with the identified protein.

* The values indicate the Mascot total protein score for the identified protein (31).

* The values indicate the Mascot total protein score confidence interval (C.I.) for the identified protein (31).
Gβγ Assembly on CCT/TRiC

FIGURE 6. Interaction of Gβ isoforms with the CCT/TRiC complex. A, FLAG-Gβ isoforms were translated in the reticulocyte lysate with unlabeled methionine and immunoprecipitated (IP) with anti-FLAG beads. The immunoprecipitates were run on an SDS-polyacrylamide gel, transferred, and separately immunoblotted (IB) with antibodies specific for the chaperonin TCP subunits α, β, γ, ε, ζ, η, and θ. Each blot was subsequently blotted with anti-FLAG antibody to visualize FLAG-Gβ. For the TCP immunoblots, only the region of the gel corresponding to 50–65 kDa is shown; the expected molecular masses of the TCP subunits are all ~60 kDa. For the FLAG immunoblots, only the region of the blot corresponding to 30–50 kDa is shown. Arrows indicate the positions of the individual TCP subunits. B, immunoblots for each chaperonin subunit (TCP) and its corresponding anti-FLAG blot were imaged with a Bio-Rad Fluor-S MAX Multimager, and the signal was quantitated. The data were normalized to reflect the relative amount of a given chaperonin subunit for the amount of each Gβ subunit present. Each experiment was done a minimum of three times, and the normalized data were averaged. Data are graphed as mean ± S.E. C, shown is a summary of the Gβ isoform interaction with the CCT/TRiC complex.

The bands for FLAG-Gγ3 and the Gβ3/FLAG-Gγ3 dimer are different. Significantly, 35S-labeled Gγ was not seen in association with the CCT/TRiC band when expressed either alone or with Gβ and forming a Gβγ dimer (lanes 7–14). These experiments demonstrate that Gβγ dimer formation can be observed on native gels and provide additional evidence for the stability of these dimers because they remain associated during electrophoresis.

The results shown in Fig. 8 indicate that dimer formation follows Gβ interaction with CCT/TRiC and is compatible with a role of CCT/TRiC in Gβγ dimer assembly. CCT/TRiC has an integral ATPase activity that has been linked to closing of the CCT/TRiC folding chamber and facilitating protein folding as well as releasing the folded substrate from the complex (21, 22, 26). This ATPase activity has also been associated with complex formation in the assembly of specific multimeric structures (35–37). The functions of this ATPase activity can be blocked by addition of adenine nucleotide analogs such as ATPγS and ADPβS or by chelation of Mg2+. In addition, the “closed” state of the CCT/TRiC complex is thought to correspond to ADP-Pi, the mid-hydrolysis state of ATP. ADP-Pi,
FIGURE 7. Gβγ dimer formation assayed on a nondenaturing gel. To determine whether nondenaturing gels could be used to monitor Gβγ dimer formation, Gβ or Gγ was independently labeled with [35S]methionine, and their mobility on 4–15% nondenaturing gels was evaluated with or without preincubation with the non-radioactively labeled complementary subunit. A: lanes 1–6, Gβγ was synthesized in the presence of [35S]methionine and combined with Gγ1 or Gγ3, that was synthesized in the absence of unlabeled methionine. [35S]-Labeled Gβγ alone (lanes 1 and 2) traveled primarily at high molecular mass. If preincubated with either unlabeled Gγ1 (lanes 3 and 4) or unlabeled Gγ3 (lanes 5 and 6), [35S]-labeled Gβγ had increased mobility dependent on with which Gγ isoform it was preincubated (arrows). Lanes 7–14, Gγ3 (lanes 7 and 8) or Gγ1 (lanes 9 and 10) was synthesized in the presence [35S]methionine; their mobility on the nondenaturing gel was isoform-specific. When unlabeled Gγ3 was preincubated with labeled Gβγ (lanes 11 and 12) or with labeled Gγ1 (lanes 13 and 14), a new band of altered mobility was observed at a similar position as observed for the [35S]-labeled Gβγ samples in the presence of unlabeled Gγ (lanes 3–6). Dimer-specific bands are indicated by arrows. B: an enlargement of the area in A shows Gγ3 alone and Gβ1-Gγ3-specific bands (arrows).

can be mimicked by ADP and by Al(NO3)3, MgCl2, and NaF, whereby the fluoride coordinates with the aluminum in a manner that mimics the γ-phosphate of ATP. Previous studies have not evaluated whether Gβγ dimer formation requires ATP independently of Gβ or Gγ synthesis. One prediction of a role of CCT/TRiC in dimer formation would be that this process is ATP-dependent; if it were not, this would suggest that the role of CCT/TRiC is primarily in the folding of Gβγ rather than participating in dimer formation directly. We therefore tested whether Gβγ dimer formation is ATP-dependent.

Gβ1, Gβ2, FLAG-Gγ1, and FLAG-Gγ3 were individually expressed with unlabeled methionine or [35S]methionine for 2 h at 37 °C and then incubated in combinations containing one unlabeled and one labeled subunit. From the time course in Fig. 8, it is apparent that any requirement for folding of Gβγ either by CCT/TRiC or other chaperones, can be completed in 60 min or less because the Gβγ dimer will have already appeared by then. In addition, in other studies, we found that productive dimer formation did not proceed beyond 2 h (data not shown). Before combining the subunits, ATP, Al(NO3)3, MgCl2, and NaF; ATPγS; or ADPβS was added to the expressed Gβ subunit (Fig. 9). Fig. 9A shows control results with 5 mM ATP. Incubation of FLAG-Gγ3 with either Gβ1 or Gβ2 resulted in generation of new [35S]-labeled Gβ bands and a slight change in the mobility of [35S]-labeled FLAG-Gγ1. Incubation of FLAG-Gγ1 with Gβ1 resulted in a new band that could be labeled with either [35S]-labeled Gβ or [35S]-labeled FLAG-Gγ1. In contrast and as expected from previous work (see “Discussion” in Ref. 12), FLAG-Gγ1 did not form a dimer with Gβ2. During dimerization in the presence of Al(NO3)3, MgCl2, and NaF (Fig. 9B); ATPγS (Fig. 9C); or ADPβS (Fig. 9D), the radioactivity associated with the dimer was abolished or greatly reduced. Conversely, the amount of [35S]-labeled Gβ migrating with CCT/TRiC was increased under these same conditions. This suggests that the Gβ involved in dimer formation came from that associated with CCT/TRiC and that this complex is the relevant target for the effect of ATP analogs on the dimerization process.

The nucleotide analogs did not promote Gγ association with CCT/TRiC, and Gβγ, a dimer that does not normally occur, was not seen either as dimer or as an intermediate of that dimer. Additional experiments showed that chelation of Mg2+ with EDTA also abolished dimer formation (data not shown).

The data in Fig. 9 suggest that CCT/TRiC mediates Gβγ dimer formation via an ATP-dependent process that involves an interaction with Gβ, but not Gγ. We tested this conclusion by evaluating whether addition of Gγ affects Gβ association with CCT/TRiC. FLAG-Gβ1 and Gγ2 were independently synthesized with unlabeled methionine, and then either excess lysate (5-fold) containing Gγ2 or mock-translated lysate (with pcDNA3) was added to lysate containing FLAG-Gβ1. After 90 min, FLAG-Gβ1 was immunoprecipitated, and samples were blotted for TCP-1 and FLAG-Gβ1 (Fig. 10). In the absence of Gγ2, TCP-1 immunostaining was more prominent than FLAG-Gβ1 staining (Fig. 10B). Immunostaining of FLAG-Gβ1 in these samples was about equal in intensity with the control (Fig. 10A) and represents immunoprecipitation of 20–30% of the synthesized FLAG protein. This is compatible with the data in Fig. 8, suggesting that, in the absence of Gγ, Gβ protein tends...
including Hsp40, Hsp70, and Hsp90. This does not negate the involvement of other proteins or cofactors, but it does suggest a primary role for CCT/TRiC in the post-translational events leading to dimer formation.

We report here substantial evidence for the role of CCT/TRiC in the folding of Gβ and as a major determinant or intermediate of Gβγ dimer formation. These include the following. 1) Gβ specifically binds CCT/TRiC (Figs. 5 and 6), a complex involved in protein folding (19, 21, 38) and protein complex formation (35, 36), shortly after its synthesis (Fig. 8); there is a lag of several minutes before dimer formation, as would be expected if folding of Gβ on CCT/TRiC is a requirement for production of functional Gβ. 2) Gβ association with CCT/TRiC is isoform-specific (Fig. 6), and the specificity of these interactions correlates closely with the ability of different Gβ isoforms to form dimers in the same rabbit reticulocyte lysate system as described here (Fig. 1) and previously (12). The selectivity of these interactions argues against nonspecific association of unfolded or misfolded, newly synthesized Gβ with CCT/TRiC and is consistent with observations that the protein substrates of this complex interact with CCT/TRiC through specific sequences (19, 23, 24). 3) We show here for the first time that Gβγ dimer formation is ATP-dependent and that this is a requirement separate from synthesis or folding of either Gβ or Gγ (Fig. 9). This is both a prediction and a test of the involvement of CCT/TRiC in dimer formation because nearly all of its activities, including folding, release of folded peptide, and complex formation, have been shown to be ATP-dependent (21, 22, 26, 35–37). That the target of this inhibition is CCT/TRiC is supported strongly by the observation that inhibition of ATPase activity leads to increased association of labeled Gβ with CCT/TRiC and is consistent with observations that the protein substrates of this complex interact with CCT/TRiC through specific sequences (19, 23, 24). 4) Addition of Gγ to previously synthesized Gβ under conditions allowing dimer formation (Fig. 1) reduces Gβ association with CCT/TRiC (Fig. 10), indicating that Gβ bound to CCT/TRiC is capable of functionally interacting with Gγ and that exposure of the complex to Gγ results in release of Gβ from CCT/TRiC.

Our results are summarized in the model shown in Fig. 11. Shortly after synthesis, Gβ associates with CCT/TRiC. This would most likely be mediated through specific sites of interaction on Gβ, as is found for most well characterized substrates (19, 21, 38), and may require partial folding of the protein, per-
happily mediated by other chaperones. The specificity of this interaction would explain the variable association of different Gβ isoforms with CCT/TRIC (Fig. 6). ATP-dependent events would then be required to mediate folding of Gβ to a state competent to bind Gγ subunits. There is evidence that, for many substrates, the folding process on CCT/TRIC proceeds through rounds of binding and ATP-dependent release, ultimately leading to properly folded native protein (39). Release of properly folded protein in the presence of Gγ subunits would allow binding of the two partners and prevent rebinding of Gβ (as a Gβγ dimer) to CCT/TRIC. This is essentially what we observed, i.e. when Gγ is added to presynthesized Gβ, it results in dissociation of Gβ from CCT/TRIC (Fig. 10). This result is comparable with three other cases in which CCT/TRIC mediates complex formation: association of the von Hippel-Lindau protein with its partner elongin B/C (33, 35), complex formation between histone deacetylase-3 and its binding partner SMRT (36), and formation of a cyclin E-CDK2 complex (37). Although the details differ somewhat between these various reactions, in all three cases, one component is folded on CCT/TRIC and is removed from the complex in an ATP-dependent reaction by its binding partner, which itself does not appear to interact with the CCT/TRIC complex.

This work explains a number of past observations about Gβ folding and Gβγ dimer assembly that include the following findings: Gβ does not fold correctly without Gγ (16, 40); prokaryotes do not have the ability to assemble Gβγ dimers (17); some in vitro systems, such as the plant wheat germ system, also lack the ability to form dimers (17); cellular requirements for dimer formation are not completely or necessarily met in in vitro assembly systems (12, 18); and there are structural requirements associated with Gβ subunits that determine their ability to efficiently form dimers (12, 18). It has been reported that the yeast Gβ homolog Ste4 associates with the TCP-1α subunit in a mass screening of interacting partners in yeast (19, 27). Our results extend this observation to animal cells and to an association with the CCT/TRIC complex rather than its isolated TCP subunits. Notably, our results complement the work of Humrich et al. (20), which showed that small interfering RNA to TCP-1α decreases Gβγ levels in human embryonic kidney 293 cells. Our studies here indicate that the requirement for TCP-1α is for folding Gβ and not for production of some other intermediate required for dimer formation. The previous experiments (20) are also important because they indicate that the mechanism we have described here using rabbit reticulocyte lysate is likely the same mechanism used by other cells.

CCT/TRIC was originally characterized in its folding of actin and tubulin (19, 21, 41, 42) and then for other proteins, including the Go subunit of transducin (39). More recently, it has been characterized in the folding of several proteins that are assembled into complexes through a CCT/TRIC-dependent mechanism (33, 35–37). The folding of these proteins, as well as any associated complex formation, can require the participation of other proteins or chaperones, and there are multiple types of co-chaperones and mechanisms associated with these
processes. In addition to the requirement for the CCT/TRiC complex shown here, Hsp90 (43) and phosducin-like protein (PhLP) (20, 44–46) may participate in Gβγ folding, the regulation of its folding, or the assembly of Gβγ dimers. In the immunoprecipitation of FLAG-Gβ or FLAG-Gγ, we did not identify any associated Hsp40, Hsp70, or Hsp90, but such interactions may be transient or unstable. The behavior of Gγ subunits on native gels (Fig. 2) seems to be largely dictated by their pI and, in contrast to Gβ, not by their interaction with other proteins. Some Gγ proteins do migrate as doublets on native gels (Fig. 2), which could indicate such interactions, but could also be explained by variable post-translational modification (32). Likewise, although the mobility of Gβ isoforms on native gels can be explained by their formation of a complex with CCT/TRiC (or by accumulation of denatured, aggregated protein on the top of the gel as in Fig. 2), there are other bands, varying in position or intensity, associated with the different isoforms. These could represent complexes with other proteins (not CCT/TRiC) involved in folding or dimer assembly.

The Gβ-binding protein PhLP has recently been reported to have a role in Gβ synthesis and Gβγ dimer assembly (20, 46). One previous study indicated that PhLP binds both Gβ and CCT/TRiC and that small interfering RNA knockdown of PhLP decreases Gβ synthesis and Gβγ dimer assembly (46). That work led to the novel conclusion that PhLP is a molecular chaperone for Gβγ dimer assembly. In contrast, another study suggested that PhLP overexpression negatively regulates Gβ synthesis and Gβγ dimer assembly by an inferred interaction with CCT/TRiC, based upon small interfering RNA knockdown of TCP-1α (a CCT/TRiC subunit), decreasing Gβ/Gβγ production (20). It is not entirely clear how to reconcile those two studies with one another. It seems probable that PhLP is one of the components (co-chaperones) of the CCT/TRiC complex involved in folding Gβ and assembling the Gβγ dimer. One possibility, perhaps compatible with both previous studies (20, 46), is that different splice variants or phosphorylated forms of PhLP reciprocally regulate these processes. It will be particularly important to work out the sequence of events involved and the relationship of PhLP to the structure of the CCT/TRiC complex. PhLP may be a permanent component of a subset of CCT/TRiC complexes, specifically targeting them to Gβ, or it may be a transient component interacting with Gβ or Gβγ at a specific stage of folding and assembly. It could be an active participant in the folding and dimer assembly process, or it could be a regulatory component. Whatever these roles are, our data indicate that the CCT/TRiC complex provides the scaffold for the events mediated by PhLP and that CCT/TRiC itself must be an active participant in the processes because PhLP is not known to possess ATPase activity, which appears to be an integral part of the dimer assembly process.

Recent studies suggest that G protein subunit isoforms extensively regulate each other’s expression in cells at both the protein and mRNA levels (47). Gβγ dimer expression is regulated both by controlled proteolysis through the proteasome (20, 48, 49) and possibly by regulation of dimer formation (20, 44). Various phosducin-like proteins have been shown to be either negative regulators of CCT/TRiC function (44, 45), suppressing Gβγ dimer expression (20), or co-chaperones in the folding of Gβ (46). The results reported here and the model in Fig. 11 provide a mechanism to explain these observations and suggest possible associated physiological consequences. In contrast to the other known protein dimers assembled by CCT/TRiC, Gβγ dimers represent a combinatorially complex family of proteins with at least 60 possible dimer combinations (7). A large number of these pairs, but not all, are biochemically compatible (Ref. 12 and references therein). One possible consequence of the model in Fig. 11 would be that the Gβ protein on CCT/TRiC provides an uncommitted reservoir of protein capable of responding to changing cellular requirements for different Gβγ dimers, dependent upon production of the small and highly variable Gγ subunit. Thus, this mechanism of Gβγ dimer production (Fig. 11) may provide a “proofreading” step whereby Gβ remains in reserve on CCT/TRiC until a correct Gγ subunit is provided to stabilize its release from the complex. Through this, the folding of Gβ by CCT/TRiC allows an additional level of regulation of the heterotrimeric G proteins based upon their inherent specificity for dimer assembly.

Acknowledgments—We thank Kevin Schey, Jennifer Bethard, John Cleator, Kathryn Robinson, Bronwyn Tatum, Lia Campbell, and Brook White for assistance and helpful discussions in the completion of this study. We also thank Drs. Henry Fong, Mel Simon, N. Gautam, William Simonds, and Nicholas Ryba for providing constructs and cDNAs. We also thank the Guthrie cDNA Resource Center (now the University of Missouri-Rolla cDNA Resource Center) for making available a number of the cDNA clones used in this study.

REFERENCES

1. International Human Genome Sequencing Consortium (2001) Nature 409, 860–921
2. Venter, J. C., Adams, M. D., Myers, E. W., Li, P. W., Mural, R. J., Sutton, G. G., Smith, H. O., Yandell, M., Evans, C. A., Holt, R. A., Gocayne, J. D., and et al. (2001) Science 291, 1304–1351
3. Birnbaumer, L. (1990) Annu. Rev. Pharmacol. Toxicol. 30, 675–705
4. Guerdmann, T., Kalkbrenner, F., and Schultz, G. (1996) Annu. Rev. Pharmacol. Toxicol. 36, 429–495
5. Gilman, A. G. (1987) Annu. Rev. Biochem. 56, 615–649
6. Brown, A. M., and Birnbaumer, L. (1996) Annu. Rev. Physiol. 52, 197–213
7. Hildebrandt, J. D. (1997) Biochem. Pharmacol. 54, 325–339
8. Neubig, R. R. (1994) FASEB J. 8, 939–946
9. Clapham, D. E., and Neer, E. J. (1997) Annu. Rev. Pharmacol. Toxicol. 37, 167–203
10. Jones, M. B., Siderovski, D. P., and Hooks, S. B. (2004) Mol. Interventions 4, 200–214
11. Hildebrandt, J. D., Codina, J., Risinger, R., and Birnbaumer, L. (1984) J. Biol. Chem. 259, 2093–2042
12. Dingus, J., Wells, C. A., Campbell, L., Cleator, J. H., Robinson, K., and Hildebrandt, J. D. (2005) Biochemistry 44, 11882–11890
13. Smith, T. F., Gaitatzes, C., Saxena, K., and Neer, E. J. (1999) Trends Biochem. Sci. 24, 181–185
14. Sondek, J., Bohm, A., Lambricht, D. G., Hamm, H. E., and Sigler, P. B. (1996) Nature 379, 369–374
15. Wall, M. A., Coleman, D. E., Lee, E., Iniguez-Lluhi, J. A., Posner, B. A., Gilmam, A. G., and Sprang, S. R. (1995) Cell 83, 1047–1058
16. Schmidt, C. J., and Neer, E. J. (1991) J. Biol. Chem. 266, 4538–4544
17. Mende, U., Schmidt, C. J., Yi, F., Spring, D. J., and Neer, E. J. (1995) J. Biol. Chem. 270, 15892–15898
18. García-Higueras, I., Gaitatzes, C., Smith, T., and Neer, E. J. (1998) J. Biol. Chem. 273, 9041–9049
19. Valpuesta, J. M., Martin-Benito, J., Gomez-Puertas, P., Carrascosa, J. L.,...
and Willison, K. R. (2002) FEBS Lett. 529, 11–16
20. Humrich, J., Bermel, C., Bunemann, M., Harmark, L., Frost, R., Quitterer, U., and Lohse, M. J. (2005) J. Biol. Chem. 280, 20042–20050
21. Hartl, F. U., and Hayer-Hartl, M. (2002) Science 295, 1852–1858
22. Frydman, J. (2001) Annu. Rev. Biochem. 70, 603–647
23. Hynes, G. M., and Willison, K. R. (2000) J. Biol. Chem. 275, 18985–18994
24. Llorca, O., McCormack, E. A., Hynes, G., Grantham, J., Cordell, J., Carrascosa, J. L., Willison, K. R., Fernandez, J. J., and Valpuesta, J. M. (1999) Nature 402, 693–696
25. Siegers, K., Bolter, B., Schwarz, J. P., Bottcher, U. M. K., Guha, S., and Hartl, F. U. (2003) EMBO J. 22, 5230–5240
26. Camasses, A., Bogdanova, A., Shevchemko, A., and Wolfgang, Z. (2003) Mol. Cell 12, 87–100
27. Ho, Y., Gruhler, A., Heilbut, A., Bader, G. D., Moore, L., Adams, S.-L., Millar, A., Taylor, P., Bennett, K., Booutilier, K., and et al. (2002) Nature 415, 180–183
28. Hansen, W. J., Cowan, N. J., and Welch, W. J. (1999) J. Cell Biol. 145, 265–277
29. Towbin, H., Staehelin, T., and Gordon, J. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 4350–4354
30. Mullikin-Kilpatrick, D., Mehta, N. D., Hildebrandt, J. D., and Treistman, S. N. (1995) Mol. Pharmacol. 47, 997–1005
31. Perkins, D. N., Pappin, D. J., Creasy, D. M., and Cottrell, J. S. (1999) Electrophoresis 20, 3551–3567
32. Cook, K. A., Schey, K. L., Wilcox, M. D., Dingus, J., Ettling, R., Nelson, T., Knapp, D. R., and Hildebrandt, J. D. (2006) Mol. Cell. Proteomics 5, 671–685
33. Hansen, W. J., Ohh, M., Moslehi, J., Kondo, K., Kaelin, W. G., and Welch, W. J. (2002) Mol. Cell. Biol. 22, 1947–1960
34. Roobol, A., and Carden, M. J. (1999) Eur. J. Cell Biol. 78, 21–32
35. Feldman, D. E., Thulasiraman, V., Ferreyra, R. G., and Frydman, J. (1999) Mol. Cell 4, 1051–1061
36. Guenther, M. G., Yu, J., Kao, G. D., Yen, T. J., and Lazar, M. A. (2002) Genes Dev. 16, 3130–3135
37. Won, K. A., Schumacher, R. J., Farr, G. W., Horwich, A. L., and Reed, S. I. (1998) Mol. Cell. Biol. 18, 7584–7589
38. Llorca, O., Matin-Benito, J., Ritco-Vonsovici, M., Willison, K. R., Carrascosa, J. L., and Valpuesta, J. M. (2000) EMBO J. 19, 5971–5979
39. Farr, G. W., Scharl, E. C., Schumacher, R. J., Sondek, S., and Horwich, A. L. (1997) Cell 89, 927–937
40. Garcia-Higuera, I., Fenoglio, I., Li, Y., Lewis, C., Panchenko, M. P., Reiner, O., Smith, T. F., and Neer, E. J. (1996) Biochemistry 35, 13985–13994
41. Cowan, N. J., and Lewis, S. A. (2002) Adv. Protein Chem. 59, 73–104
42. Dunn, A. Y., Melville, M. W., and Frydman, J. (2001) J. Struct. Biol. 135, 176–184
43. Inanobe, A., Takahashi, K., and Katada, T. (1994) J. Biochem. (Tokyo) 115, 486–492
44. McLaughlin, J. N., Thulin, C. D., Hart, S. J., Resing, K. A., Ahn, N. G., and Willardson, B. M. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 7962–7967
45. Martin-Benito, J., Bertrand, S., Hu, T., Ludtke, P. J., McLaughlin, J. N., Willardson, B. M., Carrascosa, J. L., and Valpuesta, J. M. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 17410–17415
46. Lukov, G. L., Hu, T., McLaughlin, J. N., Hamm, H. E., and Willardson, B. M. (2005) EMBO J. 24, 1965–1975
47. Krumins, A. M., and Gilman, A. G. (2006) J. Biol. Chem. 281, 10250–10262
48. Obin, M., Lee, B. Y., Meinke, G., Bohm, A., Lee, R. H., Gaudet, R., Hopp, J. A., Arshavsky, V. Y., Willardson, B. M., and Taylor, A. (2002) J. Biol. Chem. 277, 44566–44575
49. Hamilton, M. H., Cook, L. A., McRackan, T. R., Schey, K. L., and Hildebrandt, J. D. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 5081–5086