Adaptor heat shock protein complex formation regulates trafficking of the asialoglycoprotein receptor

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Adaptor heat shock protein complex formation regulates trafficking of the asialoglycoprotein receptor. Am J Physiol Gastrointest Liver Physiol 290: G369–G376, 2006. First published October 6, 2005; doi:10.1152/ajpgi.00204.2005.—In the asialoglycoprotein receptor (ASGPR) endocytic pathway, internalized receptors pass through early, recycling, and sorting endosomal compartments before returning to the cell surface. Sorting motifs in the cytoplasmic domain (CD) and protein interactions with these sequences presumably direct receptor trafficking. Previous studies have shown that association of a potential sorting heat shock protein (HSP) heterocomplex with the ASGPR-CD was regulated by casein kinase 2 (CK2)-mediated phosphorylation. Mass spectrometry and immunoblot analyses identified five of these ASGPR-CD-associated proteins as the molecular chaperones glycoprotein 96, HSP70, HSP90, cyclophilin A, and FK 506 binding protein. The present study was undertaken to determine whether any of the adaptor protein complexes (AP1, AP2, or AP3) were selectively associated with the ASGPR-CD. In conjunction with molecular chaperones, AP2 and AP1 were recovered from a CK2 phosphorylated agarose-GSH-GST-ASGPR-CD matrix. Binding of AP3 was independent of the phosphorylation status of the CD matrix. Inhibition of CK2-mediated phosphorylation with tetrabromobenzotriazole prevented AP recovery within an immunoadsorbed ASGPR complex. Rapamycin, which dissociates the HSP heterocomplex from ASGPR-CD, thereby altering receptor trafficking also, inhibited AP association. Similar results were obtained with an inhibitor of HSP90 heterocomplex formation, geldanamycin. The data presented provide evidence that recruitment of AP1 and AP2, which is necessary for appropriate receptor trafficking, is mediated by the interaction of AP with the ASGPR-CD-bound HSP complex.

casein kinase 2; adaptor protein; molecular chaperones

RECEPTOR-MEDIATED ENDOCYTOSIS, a universal mechanism for the uptake of macromolecules by cells, is initiated by the binding of the ligand to specific cell-surface receptors followed by a complex series of intracellular vesicular transfers (25). The asialoglycoprotein receptor (ASGPR) is a prototype of the class of cargo-carrying receptors that constitutively enter cells via clathrin-coated vesicles and traffic the endocytic pathway, recycling between endosomal compartments and the cell surface (44). An endocytotic trafficking mutant, Trf1, was isolated from the human hepatoblastoma cell line HuH-7 using a dual selection protocol (45). Although anterograde steps of intracellular endocytic processing of the ligand, including internalization, endosomal acidification, and degradation, were not significantly altered by the trf1 mutation, a selective subcellular redistribution of ASGPR was observed. In Trf1 cells, the surface binding of the high-affinity ASGPR ligand asialooromucoid (ASOR) was reduced by 50% and that of transferrin was reduced by 30% compared with parental HuH-7 cells without altering the absolute level of either receptor.

The gene that complemented the defect in Trf1 cells was identified as a novel isoform of the α-subunit of casein kinase 2 (CK2), designated CK2α′′ (41). CK2 is a highly conserved and ubiquitously expressed tetrameric enzyme that phosphorylates serine/threonine residues and is essential for the viability of eukaryotic cells (1, 34). The tetrameric CK2 holoenzyme consists of two α-subunits, carrying the catalytic activity, and two β-subunits, which have stabilizing and regulatory functions required for maximal activity and regulation of substrate specificity (35). A possible link between CK2α′′ expression and trafficking of the ASGPR was provided by our recent finding showing that the association of a heterocomplex of potential sorting proteins with the ASGPR-cytoplasmic domain (CD) depends on the phosphorylation of the CD acidic cluster motif (SSEEND) (19). When isolated from HuH-7 cytosol using the phosphorylated ASGPR-CD as bait, the heterocomplex was identified as members of the heat shock (HSP) and immunophilin (peptidyl-prolyl cis-trans-isomerase) families. Treatment of HuH-7 cells with the macrolide antibiotic rapamycin, known to prevent the association of immunophilin-HSP complexes (11), disrupted the HSP heterocomplex without affecting the phosphorylation status of the receptor and transformed wild-type HuH-7 cells to a mimic of the Trf1 phenotype (19).

Efficient endocytosis and trafficking of the ASGPR requires the presence of a tyrosine-based internalization motif or sorting single YXXΦ (where Φ is a bulky hydrophobic residue) that presumably interacts with one of the known adaptor protein (AP) heterotetrameric complexes (27, 38). Because phosphorylation of the tyrosine residue was shown not to be essential for faithful receptor trafficking (15), we explored the potential interaction of the various APs with HSP and the relationship to CK2-mediated phosphorylation of the ASGPR-CD as the basis to maintaining appropriate ASGPR distribution. In the present study, we provide evidence that, in conjunction with molecular chaperones, AP2, known to be localized to internalizing or early endosomes, and AP1, known to be associated with endosomes recycling to and from the trans-Golgi-network, were bound to a greater extent to the phosphorylated CD matrix. In contrast, AP3, known to be associated with late endosomes targeted to lysosomes, bound to the phosphorylated and nonphosphorylated CD equally well. Furthermore, we
showed that ATP-dependent phosphorylation of all three APs enhanced their association with the ASGPR-CD.

**MATERIALS AND METHODS**

**Cells and reagents.** HuH-7 and Trf1 human hepatoma cell lines have been described previously (41, 45, 46). Cells were cultured in RPMI 1640 (Invitrogen) containing 10% fetal bovine serum (Gemini, CA). Plasticware was obtained from BD Biosciences. Rabbit polyclonal antibody to the human ASGPR has been previously described (50). Rat monoclonal antibody (mAb) to HSP90 was obtained from Calbiochem. Mouse mAb to HSP70 was purchased from Neo Markers (Fremont, CA). Mouse mAbs to the α-subunit of AP2 (which reacts with the 105- and 110-kDa α-subunits of AP2), the γ-subunit of AP1, and the assembly protein (AP3) were obtained from Sigma. These anti-AP mAbs were recently utilized to study the role of AP in the formation of recycling vesicles (31) Tetramobromobenzotriaolozole (TBB) was a gracious gift from Dr. L. A. Finna (University of Padova, Padova, Italy). Rapamycin, galdanamycin, and other chemicals were obtained from Sigma.

**Isolation of GST-ASGPR-CD-associated proteins.** The cDNA sequence encoding the CD (MAKDFIDQILSEENHDPFQGPPPAQPLAQRLC) of the ASGPR H₂b subunit was amplified by PCR from a previously described full-length clone (33) and inserted into the EcoRI and XhoI sites of the pGStaq plasmid (Invitrogen; Carlsbad, CA) containing a thrombin cleavage site between glutathione-S-tranferase (GST) and the CD (39). Site-directed mutagenesis of the CD was performed using Gene Tailor (Invitrogen) according to the manufacturer’s instructions. pGSTaq ASGPR-CD constructs were expressed in Escherichia coli, and fusion proteins were recovered from the bacterial lysate by affinity chromatography on a glutathione-agarose column (Bio-Rad) to a polyvinylidene difluoride membrane (Immobilon-P, Millipore; Bedford, MA). Rat monoclonal antibody (mAb) to HSP90 was obtained from the manufacturer (Sigma).

**GST-ASGPR-CD fusion proteins bound to GSH-agarose beads were used as an affinity matrix to isolate potential sorting proteins from HuH-7 cytosol in the phosphorylated or nonphosphorylated state as previously described (42).**

**Binding of 125I-labeled ASOR to determine ASGPR expression.** To determine the cell surface and total expression of ASGPR, an 125I-labeled ASOR (125I-ASOR) binding assay was performed at 4°C with whole cells or with a detergent lysate as previously described (45). To measure 125I-ASOR binding, cells were chilled to 4°C on ice for 10 min and incubated with 1 µg/ml 125I-ASOR (−5,000 counts·min⁻¹·ng⁻¹) in 1.5 ml of the assay buffer at 4°C for 1 h in the absence (total binding) or presence (nonspecific binding) of 100 µg/ml unlabeled ASOR. The unbound ligand was removed, and surface-bound 125I-ASOR was determined from the radioactivity released by incubating cells for 10 min at 4°C in 1.5 ml of 20 mM EGTA in Tris-buffered saline (TBS), which caused dissociation of the Ca²⁺-dependent ligand-receptor complex (44). To determine total ASGPR expression, an asparagine binding assay was performed in detergent cell lysate. Aliquots (100–200 µl) of Triton X-100 cell lysates were added to 500 µl of assay buffer [25 mM Tris·Cl (pH 7.8), 150 mM NaCl, 20 mM CaCl₂, 0.1% (wt/vol) Triton X-100, 0.6% (wt/vol) BSA, and 1 µg 125I-ASOR] and incubated at 25°C for 30 min. The labeled complex was precipitated by the addition of an equal volume of saturated solution of ammonium sulfate and adjusted to pH 7.8 with solid Trizma base. After 10 min on ice, the suspension was filtered and washed on Whatman GF/C disks (2.4 cm) under reduced pressure to determination bound 125I-ASOR. Inclusion of 100 µg unlabeled ASOR in the assay mixture was used to determine nonspecific binding.

**Commmunoadsorption of ASGPR-associated proteins.** The UltraLink immobilized protein G-agarose gel was washed three times with PBS before samples were incubated with anti-ASGPR antibody for 1–2 h at 4°C. Unbound antibody was removed by three 5-min washes with PBS. Anti-ASGPR was covalently cross linked to the immobilized protein G using dimethyl pimelimidate according to the manufacturer’s instructions (Pierce). Cells were washed three times with ice-cold PBS (pH 7.4) and harvested in PBS containing 2% CHAPS detergent (for commmunoadsorption) with the addition of proteinase inhibitors (Sigma) and okadaic acid (1 ng/ml, Calbiochem). Insoluble material was removed by centrifugation at 20,000 g for 10 min. Protein was quantified by bicinecinonic acid (BCA) protein assay (Pierce). Equal amounts of protein from cell lysates were mixed with covalently bound antibody and incubated with constant mixing at 4°C for 1 h. Beads were washed three times with the appropriate lysis buffer and once with PBS (pH 7.4). Bound proteins were released in 2× SDS loading buffer by heating to 90°C for 10 min, resolved by 10% or 4–20% SDS-PAGE, and transferred in a semidry transfer cell (Bio-Rad) to a polyvinylidene difluoride membrane (Immobilon-P, Millipore; Bedford, MA).

**Western blot analysis.** Cell lysates, postnuclear supernatants, immunoprecipitates, or affinity matrix-purified proteins were separated by 10% or 4–20% SDS-PAGE and transferred to a polyvinylidene difluoride membrane. The membrane was blocked overnight in TBS-Tween 20 (TBST) buffer [150 mM NaCl, 50 mM Tris·HCl (pH 7.8), and 0.1% Tween 20] containing 10% nonfat dry milk. The membrane was incubated for 2 h at room temperature in one of the following antibodies: rabbit polyclonal antibody to the human ASGPR (diluted 1:5,000), rat mAb to HSP90 (diluted to 0.2 µg/ml), mouse mAb to HSP70 (diluted to 0.2 µg/ml), rabbit polyclonal antibody to FK 506 binding protein (FKBP)59/HSP56 (diluted to 1 µg/ml), or mouse mAb to AP1–3 (diluted 1:200) in TBST containing 2% dry milk. The membrane was washed five times with TBST and incubated at room temperature with the appropriate horseradish peroxidase-conjugated secondary antibody (diluted 1:5,000) in TBST containing 1% dry milk for 1 h. After being washed five times with TBST, the membrane was incubated for 1 min in chemiluminescence reagent (Perkin Elmer Life Sciences) before film exposure. The percent distribution of various proteins was determined by densitometric scan of the developed films. For sequentially probing with different antibodies, the primary antibodies were stripped with 0.1 M NaOH for 5 min at room temperature.

**Phosphate labeling of ASGPR and AP.** Cells (1 × 10⁶) preincubated at 37°C in phosphate-free MEM supplemented with 10% dialyzed FBS for 1 h were incubated with [32P]Orthophosphate (250 µCi/ml) for 3 h in the same medium with or without TBB (5 µM, 30 min before 32P addition). After being labeled, cells were washed three times with ice-cold PBS, harvested by scraping, and centrifuged at 300 g for 10 min. The cell pellet was resuspended in 0.5 ml of water, to which 2× lysis buffer [100 mM Tris (pH 7.4), 300 mM NaCl, and 2% Nonidet P-40 containing protease inhibitor mixture (Sigma) and 1 ng/ml okadaic acid] was added and maintained at 4°C for 30 min with constant mixing, followed by centrifugation at 14,000 g for 10 min. Antiserum to human ASGPR or AP2 was added to supernatants containing equal amounts of 32P-labeled proteins as determined by trichloroacetic acid precipitation. After 1-h incubation at 4°C, immobilized protein A/G (Pierce) was added, and incubation was continued for an additional 1 h. Immobilized protein A/G recovered by centrifugation at 10,000 g was washed three times with 10 mM Tris, 150 mM NaCl, 1% Triton X-100, 1% deoxycholate, and 0.1% SDS (pH 7.4) and then given a final wash with PBS. Antigen released by heating to 95°C for 3 min was resolved by 10% SDS-PAGE, and the gel was stained with Coomassie brilliant blue G 250 and fixed. The dried gels were exposed to Biomax film with an intensifying screen (Eastman Kodak) at −70°C.

**Immunofluorescence localization of ASGPR.** Nonpermeabilized cells were fixed with 4% paraformaldehyde and incubated for 1 h with rabbit polyclonal antibody to the human ASGPR (diluted 1:200). After cells were washed with PBS, the deposition of primary antibody was detected by incubation with 1:100 fluorescein isothiocyanate-labeled goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories; West Grove, PA) for 1 h. Images were acquired on an Olympus IX70 fluorescent microscope equipped with a charge-coupled device digital camera.
camera. Digital images were assembled and labeled using Scion Image (Scion; Frederick, MD) and Adobe PhotoShop (Adobe Systems).

RESULTS

Enhanced AP binding to phosphorylated ASGPR-CD. Previously, it has been shown that association of a HSP-immunophilin complex with the CD of ASGPR was dependent on the phosphorylation status of the CK2 acidic cluster (SSEEND) motif (19). With the use of the same approach, a GST fusion protein with the full-length CD of the ASGPR H2b subunit containing both the acidic cluster and the phenylalanine-substituted tyrosine internalization motif (FQDI) was bound to GSH-agarose beads as an affinity matrix. To isolate and identify associated APs, cytosol obtained from HuH-7 cells was passed through the matrix with or without prior in vitro phosphorylation of the GST-ASGPR-CD by CK2. Previously, it has been reported that, under these conditions, the phosphorylation status of the GST-CD was unchanged by the passage of cytosol (19). In a fashion similar to that previously seen for the HSP complex (19) and repeated here as an internal positive control, phosphorylation of the CD acidic cluster enhanced the recovery of AP1 and AP2 (Fig. 1). In contrast, binding of the closely related AP3 complex was independent of the CD phosphorylation status, indicating that the enhancement of AP1 and AP2 binding to the phosphorylated ASGPR-CD was specific. In the absence of the ASGPR-CD sequence, APs were not recovered in the elution fraction. Site-directed mutation of the CK2 acidic cluster indicated that phosphorylation of either serine was sufficient to establish HSP and AP binding with the ASGPR-CD (Fig. 2). Disruption of the CK2 motif by alanine replacement of either both serine or glutamic acid residues substantially inhibited HSP and AP association with the ASGPR-CD.

Immunoadsorption of AP-ASGPR complexes. As an alternative approach to detect the potential association of AP with ASGPR in a more physiological state, HuH-7 cells were lysed with the mild detergent CHAPS in an attempt to retain protein-protein interactions (26), and the receptor and any associated proteins were coimmunoadsorbed to covalently bound anti-ASGPR. Because it has been previously shown that phosphorylated APs have higher affinities for tyrosine-based internalization motifs (12, 30), ATP (1 mM) was added to the lysate in the presence of the phosphatase inhibitor okadaic acid before coimmunoadsorption. In addition to ATP, AMP, the nonhydrolysable ATP analog adenylyl imidodiphosphate (AMP-PNP), or GTP was added to cell lysates at 1 mM before coimmunoadsorption of potential receptor AP complexes (Fig. 3). As

Fig. 1. Adaptor protein (AP) association with the asialoglycoprotein receptor (ASGPR) cytoplasmic domain (CD) is enhanced by casein kinase 2 (CK2)-mediated phosphorylation. Cytosol from HuH7 cells was passed through a glutathione-S-transferase (GST)-ASGPR-CD glutathione-agarose bead affinity matrix without (CD) or with phosphorylation (CDP) of the CD by CK2. Columns were washed with PBS, and bound proteins eluted with PBS were adjusted to 1.0 M NaCl for resolution by 4–20% SDS-PAGE. APs (AP1–3) were identified by immunoblot analysis as described in MATERIALS AND METHODS. This is a representative study of 3 independent experiments. HSP, heat shock protein.

Fig. 2. Site-directed mutation of the CK2 acidic cluster. Cytosol from HuH-7 cells was passed through the GST-ASGPR-mutated CD glutathione-agarose bead affinity matrix preincubated with CK2. Columns were washed with PBS, and bound proteins eluted with PBS were adjusted to 1.0 M NaCl for resolution by 4–20% SDS-PAGE. HSP90, HSP70, AP1, and AP2 were identified by immunoblot analysis as described in MATERIALS AND METHODS. This is a representative study of 3 independent experiments.

Fig. 3. Association with of APs with the ASGPR-CD is enhanced by ATP addition. Anti-ASGPR covalently fixed to Sepharose-protein G was used to coimmunoadsorb ASGPR-associated proteins from HuH-7 cell lysate incubated at either 4 or 37°C for 30 min in the presence of various nucleotides (1 mM). Immunoadsorbed proteins were resolved by SDS-PAGE, and the extent of AP recovery was determined by immunoblot analysis as described in MATERIALS AND METHODS. Data presented are representative of 5 independent experiments. AMP-PNP, adenylyl imidodiphosphate.
expected for an enzymatic reaction, incubation at 4°C did not enhance the binding of APs to ASGPR (data not shown). Unlike most kinases, both ATP and GTP can be utilized for CK2-mediated phosphorylation (28). However, only ATP preincubation markedly increased the amount of AP1, AP2, and AP3 recovered after incubation at 37°C (Table 1). When normalized to recovered ASGPR, the addition of ATP increased AP1, AP2, and AP3 recovery by almost threefold over the control level seen in the absence of added nucleotide. The effect of GTP addition on the enhancement of AP1 and AP2 recovery was much less substantial than that seen with ATP, which enhanced AP1 recovery by only twofold over the control level. Unlike AP1 and AP2, the addition of GTP had no apparent effect on AP3 association with the receptor. The addition of the various nucleotides to the cell lysates had no effect on the recovery of ASGPR.

Selective inhibition of CK2. In an attempt to sort out the potential roles for CK2 and an alternative kinase in mediating enhanced recovery of AP with ASGPR, a specific inhibitor of CK2 (40), TBB, was added to the coimmunoadsorption assay (Fig. 4A). Inclusion of TBB in the cell lysate before the ATP addition inhibited the coimmunoadsorption of both AP1 and AP2 with ASGPR (Table 1). Consistent with the findings shown in Fig. 1, the addition of TBB did not inhibit the coimmunoadsorption of AP3, suggesting that the enhanced recovery of AP3 in the presence of ATP was independent of CK2 activity. To confirm that the inhibitory activity of TBB was limited to CK2-mediated phosphorylation of the receptor and did not affect AP phosphorylation, ASGPR and AP were metabolically labeled with [32P]orthophosphate in HuH-7 cells in the presence or absence of TBB (Fig. 4B). Consistent with the selective inhibition of CK2 by TBB, the incorporation of 32P was reduced by almost sixfold in ASGPR with no effect on the phosphorylation of the ~50-kDa μ-subunit of AP2. Phosphorylation of the other AP2 subunits was barely detectable under these conditions (data not shown). In agreement with specific inhibition of CK2 by TBB was the reduced recovery of HSP associated with ASGPR immunoprecipitated from lysates of TBB-treated HuH-7 cell (Fig. 4C). The reduction of HSP binding seen in the presence of the inhibitor was similar to that seen in the CK2α'-deficient trf1 mutant (Fig. 4C).

To determine whether the reduction of CK2 activity and the subsequent reduction of AP association was coincident with altered ASGPR trafficking, HuH-7 cells were treated with TBB for 2 h, and cell surface and total expression of ASGPR, as measured by 125I-ASOR binding, were determined (Fig. 5). Cell surface expression of ASGPR in cells treated with TBB was reduced by almost 45%. Total ASGPR binding activity was unaffected by TBB treatment. Consistent with the specific

### Table 1. Recovery of APs and HSPs from HuH-7 cell lysates

<table>
<thead>
<tr>
<th>Recovered Protein</th>
<th>AMP</th>
<th>ATP</th>
<th>AMP-PNP</th>
<th>GTP</th>
<th>ATP + TBB</th>
<th>Rapamycin (0.08 mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AP1</td>
<td>115±7</td>
<td>1130±40</td>
<td>102±6</td>
<td>420±15</td>
<td>90±7</td>
<td>8±2</td>
</tr>
<tr>
<td>AP2</td>
<td>102±9</td>
<td>602±30</td>
<td>90±2</td>
<td>261±20</td>
<td>120±9</td>
<td>20±7</td>
</tr>
<tr>
<td>AP3</td>
<td>111±12</td>
<td>701±10</td>
<td>130±12</td>
<td>84±7</td>
<td>140±20</td>
<td></td>
</tr>
<tr>
<td>HSP90</td>
<td>803±30</td>
<td>102±6</td>
<td>102±6</td>
<td>30±5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HSP70</td>
<td>611±22</td>
<td>88±3</td>
<td>10±3</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SD (expressed as relative recovery as %control) of 3 or more independent experiments. There was no addition of cell lysate to the control. AP, adaptor protein; HSP, heat shock protein; TBB, tetrabromobenzotriazole. Values for AP2 are sums of the two bands recovered.
The effect of TBB, when the trf1 mutant (which is defective in CK2 expression) was treated with the inhibitor, there was no additional redistribution of surface ASGPR. The full ASGPR activity recovered in cell lysates indicates that TBB inhibition of CK2 activity resulted in an intracellular redistribution of the ASGPR without altering total receptor expression. Therefore, the treatment with TBB mimicked the trf1 mutation, confirming our previous finding that the Trf1 phenotype resulted from a deficit in CK2 activity and that association of the HSP complex requires CK2-mediated phosphorylation of the ASGPR-CD (41).

Cell surface ASGPR expression was measured independently of its binding activity (Fig. 6) to determine whether the observed loss of ASOR binding activity was the result of an inhibition of trafficking or the selective inactivation of plasma membrane-associated receptors. TBB-induced reduction in plasma membrane ASGPR content of HuH-7 cells was confirmed by immunofluorescent microscopic localization of the receptor on nonpermeabilized cells. In agreement with the biochemical data, treatment of HuH-7 cells markedly reduced immunodetectable plasma membrane-associated ASGPR without affecting the distribution of ASGPR in Trf1 cells.

Inhibition of HSP complex formation reduces AP binding.

To further resolve the effect of the ASGPR-CD phosphorylation state on the association of APs, HuH-7 cells were treated with increasing concentrations of the macrolide antibiotic rapamycin, which is known to prevent the association of HSP-immunophilin complexes (51), or the benzoquinone ansamycin geldanamycin, which specifically inhibits HSP90 protein-protein interactions (37). Coincident with dissociation of the HSP complex, rapamycin was previously shown to reduce cell surface ASGPR without changing the phosphorylation status or absolute level of the receptor (19). The addition of increasing concentrations of rapamycin to cell lysates before the coinmunoadsorption of ASGPR-AP showed that both AP1 and AP2 association with the ASGPR-CD were dramatically reduced with the dissociation of the HSP complex (Fig. 7 and Table 1).

Table 1. Although the response to increasing concentrations of rapamycin was not strictly linear, suggesting the possibility that other protein-protein interactions were involved in AP binding to ASGPR-CD, this finding strongly supports the notion that it is the association of the HSP complex and not the phosphorylation status of the ASGPR-CD per se that is required for immunorecovery of AP. To determine whether geldanamycin treatment altered surface expression of ASGPR...
in a fashion similar to rapamycin, HuH-7 cells were exposed to geldanamycin for 1 h at either 4 or 37°C. As shown in Fig. 8, geldanamycin reduced surface ASGPR by 42% without any notable effect on total receptor expression. That the reduction was temperature dependent suggests that active ASGPR trafficking was necessary for the geldanamycin-induced redistribution of the receptor. The requirement of HSP for the formation of a stable ASGPR-AP complex gained further support by the addition of anti-HSP antibody before the addition of ATP and the subsequent recovery of the ASGPR-AP immunocomplex. As shown in Fig. 9, immunodepletion of HSP by the addition of either anti-HSP90 or anti-HSP70 mAbs substantially reduced AP recovery with immunoabsorbed ASGPR compared to the control, to which nonimmune mouse IgG had been added.

**DISCUSSION**

In our previous report (45), a membrane protein trafficking mutant (Trf1) derived from the human hepatoma cell line HuH-7 was shown to be defective in the cell surface trafficking of receptors for asialoorsomucoid, transferrin, and mannose-terminating glycoproteins. The gene that complemented the Trf1 phenotype was shown to be a novel α-subunit of CK2 designated CK2α' (41). It is 91% identical in amino acid sequence to previously described CK2α (24). A comparison of the phosphorylation status of ASGPR in Trf1 and HuH-7 cells indicated that the trf1 mutation results in ASGPR hypophosphorylation (41). The identification of serine as the major phosphoamino acid in the ASGPR (33) and the presence of a CK2 phosphorylation motif (SSEEND) in the CD of the human ASGPR were consistent with a direct role for CK2 in trafficking of this receptor. Using CK2-phosphorylated ASGPR-CD as bait, we isolated a heterocomplex of potential sorting proteins comprising members of both the HSP and immunophilin families. The gene that complemented the Trf1 phenotype was shown to be a novel α-subunit of CK2 designated CK2α’ (41). It is 91% identical in amino acid sequence to previously described CK2α (24). A comparison of the phosphorylation status of ASGPR in Trf1 and HuH-7 cells indicated that the trf1 mutation results in ASGPR hypophosphorylation (41). The identification of serine as the major phosphoamino acid in the ASGPR (33) and the presence of a CK2 phosphorylation motif (SSEEND) in the CD of the human ASGPR were consistent with a direct role for CK2 in trafficking of this receptor. Using CK2-phosphorylated ASGPR-CD as bait, we isolated a heterocomplex of potential sorting proteins comprising members of both the HSP and immunophilin families. These HSPs are well-recognized molecular chaperones that are required for the proper folding and trafficking of many proteins (9, 30).

The marked increase in AP association with ASGPR after the addition of ATP to the cell lysate and incubation at 37°C was strongly suggestive that an enzymatic phosphorylation of either APs or ASGPR-CD enhanced the protein-protein interaction. On the basis of the GST-ASGPR-CD pulldown results and the consistent findings of increased AP association with CK2-phosphorylated CDs of diverse membrane proteins (16, 20, 23, 49), it seemed reasonable to hypothesis that CK2 phosphorylation of the ASGPR-CD was responsible for the observed enhancement of AP binding and would be consistent with the recovery of CK2 activity in clathrin-coated vesicle preparations (13, 21). This role for CK2 was further supported by the observed reduction of immunorecovered APs from cell lysates to which TBB, a specific CK2 inhibitor (40), was added before ATP addition.

Contrary to this rather straightforward explanation for increased ASGPR-AP association, the substitution of GTP for ATP, which can be utilized equally well for CK2-mediated phosphorylation (28), did not increase immunorecovered APs to the same level observed when ATP was added to cell lysates (29% and 42% recovery of AP1 and AP2, respectively). Therefore, it would seem that, whereas CK2-mediated phosphorylation of the ASGPR-CD might be necessary to augment AP binding (2), it is not sufficient to either form a stable complex or to bring AP binding to the maximum level seen with the addition of ATP (5). Short of a trivial explanation for the failure of GTP to elicit the same level of enhanced AP recovery, such as an increased rate of GTP hydrolysis in HuH-7 cell lysate, these results suggested that an ATP-dependent kinase in addition to CK2 might also be involved in AP-ASGPR-CD association. Recently described adaptor-associated kinases (AAK1) (10), which selectivity phosphorylate the AP2 μ2-subunit or cyclin G-associated kinase [GAK/auxilin-2; which phosphorylates the μ1-subunit of AP1 (16)], resulting in high-affinity binding of AP1 (17) and AP2 (12) to membrane protein sorting signals, such as the tyrosine/phenylalanine-based sorting signal of ASGPR (44), are likely candidates for this additional kinase activity.

Treatment of parental HuH-7 cells with the macrolide antibiotic rapamycin, which dissociates the HSP complex, mimicked the Trf1 phenotype by decreasing cell surface ASGPR by ~50% without affecting total receptor expression or phosphorylation (19). Analysis of the ASGPR-CD heterocomplex after rapamycin treatment indicated that the antibiotic prevented the formation or disrupted the association of HSP with the...
ASGPR. The addition of rapamycin to cell lysates before the coimmunoabsorption of ASGPR-AP dramatically reduced both AP1 and AP2 association with the ASGPR in line with the dissociation of the HSP complex (8). This finding suggested that it was the association of the HSP complex and not the phosphorylation status of the ASGPR-CD per se that was required for AP binding. The similarity of TBB treatment to that of rapamycin, both in the reduction of AP and HSP association with ASGPR and the redistribution of the cell surface receptor, indicates a functional link between ASGPR trafficking and these associated proteins. The temperature-dependent effect of geldanamycin suggests this to be a dynamic process of multiple protein-protein interactions dependent on HSP90 association with the ASGPR-CD during active receptor trafficking. To explore the sequence of binding events, attempts were made to coimmunoprecipitate an AP-HSP complex from cytosol in the absence of ASGPR. A number of standard coimmunoprecipitation protocols were applied without success (data not shown), suggesting that the AP-HSP complex was either unstable or not preformed before association with the ASGPR-CD in a ternary complex. We also screened for the presence of heat shock cognate protein 70, as a potential member of the ternary complex, without success (data not shown). However, in support of a significant role for HSPs in AP association, we were able to demonstrate that the addition of anti-HSP90 or anti-HSP70 antibodies before immunoadsorption of ASGPR interfered with AP binding. Taken together, these results suggest a sequence of events in which the ASGPR-CD first associates with HSP before the addition of AP, thereby forming the appropriate trafficking complex.

Although the functional significance of the CK2 site on ASGPR has not been established, phosphorylation of the CK2 site was shown to be necessary for 46-kDa mannose 6-phosphate receptor trafficking to the plasma membrane (7), which is consistent with the Trf1 phenotype and the observed effects of TBB or rapamycin on ASGPR distribution. However, a number of direct binding studies have shown that AP1 will associate with the mannos 6-phosphate receptor CD without prior phosphorylation of its CK2 motif (18, 47). The appropriate trafficking of the transferrin and LDL receptors and ASGPR have been shown to be dependent on either AP1 or AP2 interaction with the tyrosine-based internalization signal (14, 48). The diversity of membrane proteins affected by the tryfl mutation and the common alteration in their distribution, i.e., approximately a 50% reduction of surface expression, suggests that the association of the HSP complex mediated by CK2 phosphorylation could play a regulatory role in AP-directed trafficking and provide the common mechanism for the pleiotropic phenotype of the tryfl mutation.

Trafficking of ASGPR-containing vesicles along the microtubular network is well established (3). Recovery of immunophilins within the ASGPR-CD heterocomplex was consistent with previous reports demonstrating that steroid receptor heterocomplexes often contain several proteins that possess tetratricopeptide repeats, which are degenerative sequences of 34 amino acids involved in protein-protein interactions (6). Such proteins include members of the immunophilin family, such as recovered FKBP and cyclosporin A-binding protein. The presence of FKBP in the ASGPR-HSP90 heterocomplex, similar to that in the glucocorticoid receptor HSP90 complex (43), has been suggested to provide a protein link to the molecular motor dynein for minus end movement along microtubules. Although several previous studies have suggested that dynein plays a role in late endosome motility (22, 29, 36, 52), our recent studies directly demonstrate that dynein is associated with ASGPR-depleted vesicles (4) and may not be part of the receptor HSP-AP complex. Nevertheless, a CK2-driven phosphorylation/dephosphorylation cycle could mediate the necessary protein-protein interactions of the ASGPR-HSP-immunophilin complex with the tyrosine-based signal recognition AP1/clathrin coat recently shown to be necessary for the formation of ASGPR-containing recycling vesicles (32). Regulating the dynamics of these combinations would establish the appropriate trafficking pattern and the steady-state distribution of the ASGPR and other membrane proteins with tyrosine-based sorting motifs.

GRANTS

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REFERENCES


