Phosphorylation-dependent Interaction of the Asialoglycoprotein Receptor with Molecular Chaperones*

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A membrane protein trafficking mutant (Trf1) of HuH-7 alters the asialoglycoprotein (ASGPR) and transferrin receptor subcellular distribution. Expression cloning of a cDNA complementing the trf1 mutation led to the discovery of a novel casein kinase 2 catalytic subunit (CK2α’’). To purify potential CK2α’’ phosphorylation-dependent sorting proteins from cytosol, the ASGPR cytoplasmic domain was expressed as a GST fusion protein and immobilized on glutathione-agarose. In the absence of phosphorylation, only trace amounts of cytosol protein were bound and eluted. When the fusion protein was phosphorylated, a heterocomplex of potential sorting proteins was recovered. Mass spectrometer and immunoblot analysis identified five of these proteins as gp96, HSP70, HSP90, cyclophilin-A, and FKBP18. Treatment of HuH-7 with rapamycin to disrupt the heterocomplex reduced surface ASGPR binding activity by 55.7%. In Trf1 cells, surface-binding activity was 48 ± 7% of that in HuH-7 and was not further reduced by rapamycin treatment. Immunoblot analysis showed significantly fewer surface receptors on rapamycin-treated HuH7 cells than on nontreated cells, with no effect on the level of surface receptors in Trf1 cells. The data presented provide evidence that phosphorylation of the ASGPR cytoplasmic domain is required for the binding of specific molecular chaperones with the potential to regulate receptor trafficking.

Endocytosis is a process by which the cell internalizes proteins and lipids from the plasma membrane and transports them through a variety of tubular-vesicular compartments (1). Receptor-mediated endocytosis is distinguished from other forms of endocytosis by the initial binding of ligand to cargo-carrying receptors prior to internalization through the formation of an endocytic vesicle. ASGPR1 is a well-characterized cargo-carrying receptor that constitutively enters cells via clathrin-coated pits and traffics the endocytic pathway, recycling between early endosomal compartments and the cell surface (2). Recently, Trf1, an endocytic trafficking mutant, was isolated from the human hepatoblastoma cell line HuH-7 using a dual selection protocol (3). Although anterograde steps of intracellular endocytic processing of ligand, including internalization, endosomal acidification, and degradation, were not significantly altered by the trf1 mutation, a selective redistribution of ASGPR was observed. In Trf1 cells, the surface binding of the high affinity ASGPR ligand, asialo-oromucoid (ASOR), was reduced by 50% and that of transferrin by 30% compared with parental HuH-7 cells without affecting total receptor levels. In addition, the mutation expressed by Trf1 cells reduced the surface expression of several unrelated membrane proteins including connexin 43 (4).

Expression cloning identified the gene that complemented the defect in Trf1 cells as a novel isoform of the α-subunit of CK2, designated CK2α’’ (5). CK2 is a highly conserved and ubiquitously expressed tetrameric enzyme that phosphorylates serine/threonine residues and is essential for the viability of eukaryotic cells (6, 7). 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 (8). The newly described CK2α’’ is 91% identical in the amino acid sequence, the last 32 being unique, and 65.2% identical in the nucleotide sequence to the previously described CK2α (9). Based on the results of 32P labeling, it was evident that ASGPR expressed by Trf1 cells was hypophosphorylated compared with ASGPR expressed by the parental HuH-7 cell line (3). The identification of serine as the major phosphoamino acid in ASGPR (10) and the presence of a CK2 phosphorylation motif (SSEEND) in the cytoplasmic domain (CD) of ASGPR, are consistent with a potential role for CK2 in receptor trafficking. Indeed, of the endocytic receptors affected by the trf1 mutation including the ASGPR, the transferrin receptor, and the mannose receptor, all possess a CK2 consensus motif in their CDs.

In the present study, a heterocomplex of potential sorting proteins associated with the ASGPR-CD that depends on CK2 phosphorylation of its acidic cluster motif were isolated and identified as members of the heat shock 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 heterocomplex, resulting in the transformation of the wild type to a mimic of the Trf1 phenotype.

EXPERIMENTAL PROCEDURES

Cells and Reagents—The HuH7 and Trf1 human hepatoma cell lines were described previously (3–5). Cells were cultured in RPMI 1640 (Invitrogen), containing 10% fetal bovine serum (Gemini, CA). Plasticware was from BD Biosciences. The rabbit polyclonal antibody to the human ASGPR was previously described (12). The rat monoclonal an-
tibody (mAb) to HSP90 was obtained from Calbiochem. The mouse mAb to HSP70 was purchased from Neo Markers (Fremont, CA). The rabbit polyclonal antibody to FKBP58/HSP56 was obtained from Affinity BioReagents, Inc. (Golden, CO). Raptamycin and other chemicals were obtained from Sigma Chemicals.

**Isolation of GST-ASGPR-CD-associated Proteins**—The cDNA sequence encoding the cytoplasmic domain (MAKFDQDFIQQSLSEEENDH-PFHQGQPPAQPLARLQ) of the ASGPR H₂ subunit was amplified by PCR from a previously described full-length clone (10) and inserted into the EcoRI and Xhol sites of pGSTaq plasmid containing a thrombin cleavage site between GST and the CD (13). The pGSTaq ASGPR-CD construct was expressed in *Escherichia coli*, and the fusion protein was recovered from the bacteria lysate on a glutathione (GSH) affinity matrix as outlined by the manufacturer (Sigma). The GST-ASGPR-CD fusion protein bound to GSH-agarose beads was used as an affinity matrix to isolate potential sorting proteins from the cytosol. Phosphorylation of the GST-ASGPR-CD fusion protein with CK2 (Sigma) was performed while it was immobilized on the GSH-agarose beads. Beads (20 μl, containing 10–20 μg of fusion protein) were washed with CK2 reaction buffer (20 mM Tris-HCl, pH 7.5, 50 mM KCl, 10 mM MgCl₂). Beads were resuspended in 50 μl of reaction buffer containing 2 units/ml CK2 (human recombinant from *E. coli*, New England BioLabs Inc., Beverly, MA) and ATP (200 μM) for 2 h at 30 °C. The unincorporated phosphate and enzyme were removed by washing the beads three times with CK2 reaction buffer and incubation with the same buffer for 1 h at 37 °C. Cytosol isolated from three 100-mm plates of confluent HuH-7 cells was passed through bound GST-ASGPR-CD with or without prior phosphorylation. Columns were washed three times with PBS (pH 7.4), and bound proteins were eluted with 1.0 M NaCl. Eluted proteins were concentrated by the addition of an equal volume of 20% trichloroacetic acid prior to centrifugation at 20,000 × g for 10 min. The pellet was dissolved in 2× SDS sample buffer and the pH adjusted to an alkaline range with NH₄OH for resolution on SDS-PAGE.

**Mass Spectrometry Analysis**—The Coomassie Blue-stained bands were excised from SDS-polyacrylamide gels and completely destained with 200 mM ammonium bicarbonate in 50% acetonitrile. These gel pieces were treated with 10 mM dithiothreitol in 0.1 M ammonium bicarbonate for protein reduction. Free cysteine residues were alkylated with freshly made 55 mM iodoacetamide in 0.1 M ammonium bicarbonate. Tryptic digestion was started with the addition of 25 ng of trypsin (Promega) in ammonium bicarbonate buffer. The protein was digested for at least 16 h at 30 °C with agitation. The digestion products were cleaned and concentrated using micro-C18 ZipTips (Millipore), mixed with 0.5 μl of 10 mg/ml 1-cyano-4-hydroxysuccinimnic acid in 50% acetonitrile, 0.1% (v/v) TFA, and applied onto a Maltide plate. MALDI mass spectra were recorded with a PerSeptive Voyager-DE STR MALDI time-of-flight mass spectrometer operated in the reflectron mode. The mass measurement accuracy with internal calibration was, in general, better than 100 ppm. The measured peptide masses were used for data base searching with Profound algorithm (ProteoMetrics, NY).

**Cell Surface Binding of 125I-ASOR**—Confluent cells in 60-mm dishes were incubated with 125I-ASOR assay buffer (135 mM NaCl, 0.81 mM MgSO₄, 1.2 mM MgCl₂, 27.8 mM glucose, 2.5 mM CaCl₂, 25 mM HEPES, pH 7.2). After washing, the cells were preincubated at 37 °C for 1 h. To measure 125I-ASOR binding, cells were chilled to 4 °C for 10 min and incubated with 1 μg/ml 125I-ASOR 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. Unbound ligand was removed by washing with assay buffer. 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 TBS, causing dissociation of the Ca²⁺-dependent ligand-receptor complex.

**Biotinylation of Cell Surface Proteins**—Confluent cells were washed twice with prewarmed ASOR assay buffer and incubated with the same assay buffer for 1 h at 37 °C followed by storage on ice for 20 min. The prechilled cells were washed twice with ice-cold PBS (pH 8.0) prior to a 1-h incubation on ice with 2 ml of the membrane-impermeant label EZ-Link™ Sulfo-NHS-Biotin (0.5 mg/ml, sulfosuccinimidobiotin obtained from Pierce) in PBS (pH 8.0). The reaction was stopped by adding glycine to a final concentration of 1 mg/ml for 2 min on ice. Cells were washed three times with ice-cold PBS (pH 7.4) and harvested by scraping, and the protein was extracted with 150 mM NaCl/0.1% SDS sample buffer by heating to 90 °C for 10 min, resolved by 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, post-nuclear supernatants, immunoprecipitated or affinity matrix-purified proteins, were resolved on 10 or 4–20% SDS-PAGE and transferred to a polyvinylidene difluoride membrane. The membrane was blocked overnight in TBST buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 0.1% Tween 20) containing 1% nonfat dry milk. The membrane was incubated for 2 h at room temperature in the appropriate HRP-conjugated secondary antibody (diluted to 1:500) in TBS containing 1% dry milk for 1 h. After being washed 5 times with TBST, the membrane was incubated for 1 min in chemiluminescence reagents (PerkinElmer Life Sciences) prior to film exposure. For sequentially probing with different antibodies, the primary antibodies were stripped with 0.1 M NaOH for 5 min at room temperature.

**Fluorescence Localization of ASGPR**—Nonpermeabilized cells were fixed with 4% paraformaldehyde and incubated with 1 h with rabbit polyclonal antibody to the human ASGPR (diluted to 1:200). Following washes 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 CCD digital camera. Digital images were assembled and labeled using Scion Image (Scion Corp., Frederick, MD) and Adobe Photoshop (Adobe Systems Inc.).

**RESULTS**

**Isolation and Identification of Phosphorylated Cytoplasmic Domain-associated Proteins**—The endocytic trafficking mutant (Trf1) isolated from HuH7 cells is defective in cell surface ASGPR expression (3). This defect is the result of the failure of Trf1 to express a novel isoform of CK2 α (CK2 α’) resulting in the hypophosphorylation of the ASGPR (5). Because it is reasonable to assume that ASGPR intracellular trafficking is directed by soluble sorting proteins that bind to the cytoplasmic domain (CD) of the receptor, a GST fusion protein with the full-length CD of the ASGPR H₂b subunit (10) was constructed to isolate the putative sorting complex. The GST-ASGPR-CD fusion protein was bound to GSH-agarose beads that were used as an affinity matrix to isolate potential sorting proteins from cytosol. Because the ASGPR-CD contains a CK2 phosphorylation site (S/S/EEN), it could be selectively labeled (Fig 1). In the absence of added enzyme, no 32P was incorporated into the fusion protein. Cytosol not shown. Cytosol was passed through the bead matrix with or without prior *in vitro* phosphorylation of GST-ASGPR-CD by CK2. The affinity matrix washed with PBS, and bound proteins were eluted with 1.0 × NaCl. Isolated proteins were concentrated and resolved on SDS-PAGE. As shown in figure 2, in the absence of phosphorylation only trace amounts of protein were bound and eluted from the GST-ASGPR-CD fusion protein affinity matrix. These proteins appeared to be nonspecific, as they were often recovered...
Cytosol from HuH7 cells was passed through the GST-ASGPR-CD glutathione-agarose bead affinity matrix (40 units/ml) in 150 mM NaCl, 50 mM Tris-HCl, pH 8.0, 2.5 mM CaCl2. Bound fusion protein was digested at the cleavage site with thrombin with PBS and bound proteins eluted with PBS adjusted to 1 M NaCl for GST-CD fusion protein (10 mM dithiothreitol. Following heat denaturation, the incubation mixture was resolved on 15% SDS-PAGE. Partial digestion of GST-CD resulted in three bands detected by Coomassie Blue staining and two bands by 32P incorporation. The absence of 32P in GST indicates a selective labeling of the CD fragment.

**FIG. 1.** Phosphorylation of GST-CD fusion protein by CKII. GST-CD fusion protein (10 µg) bound on glutathione-agarose beads was labeled by incubation at 37 °C for 30 min with CK2 (2 units/ml) in 50 µl of CK2 assay buffer, described under “Experimental Procedures,” containing [γ-32P]ATP (10 µCi). To define the site of 32P incorporation, the bound fusion protein was digested at the cleavage site with thrombin (40 units/ml) in 150 mM NaCl, 50 mM Tris-HCl, pH 8.0, 2.5 mM CaCl2, 1 mM dithiothreitol. Following heat denaturation, the incubation mixture was resolved on 15% SDS-PAGE. Partial digestion of GST-CD resulted in three bands detected by Coomassie Blue staining and two bands by 32P incorporation. The absence of 32P in GST indicates a selective labeling of the CD fragment.

**FIG. 2.** Isolation and identification of ASGPR cytoplasmic domain-associated proteins. Cytosol from HuH7 cells was passed through the GST-ASGPR-CD glutathione-agarose bead affinity matrix with (CD-P) or without phosphorylation by CK2. Columns were washed with PBS and bound proteins eluted with PBS adjusted to 1 M NaCl for resolution on 4–20% SDS-PAGE. Protein bands identified with Coomassie Blue staining (as shown) were excised and digested with trypsin for analysis by mass spectrometer. Proteins were confirmed (*) or identified (**) by Western blot analysis (see insert) as described under “Experimental Procedures.” MW, prestained molecular weight standards.

**FIG. 3.** Surface binding of ASOR is reduced by rapamycin. Confluent HuH-7 cells were treated with increasing concentrations of rapamycin for 24 h, and cell surface 125I-ASOR binding was measured at 4 °C as described under “Experimental Procedures.” Data represent the mean ± S.D. of four independent experiments. The results indicate that the reduction of cell surface ASGPR activity induced by rapamycin was dose-dependent.

Because it has been shown that the macrolide antibiotic rapamycin by virtue of its binding to FKBP's may dissociate HSP-immunophilin heterocomplexes (14), HuH7 and Trf1 cells were treated with rapamycin, and cell surface expression of ASGPR, as measured by 125I-ASOR binding activity, was determined. Rapamycin treatment reduced surface binding of ASOR in HuH7 cells in a dose-dependent fashion, reaching a minimum of 45 ± 5.7% at 3 µM for 24 h (Fig. 3). Inhibition of ASOR binding by the antibiotic was also time-dependent, reaching a maximum of 48 ± 3% of control within 3 h at 1 µM, with an initial 28 ± 5% reduction of binding activity evident as early as 2 h (Fig. 4). In contrast to HuH-7 cells, cell surface ASOR binding was not reduced in rapamycin-treated Trf1 cells (Fig. 4) even when treatment was extended to 24 h at 3 µM (data not shown).

To determine whether the loss of ASOR binding activity was the result of an inhibition of trafficking or inactivation of cell surface receptors, ASGPR expression was measured independently of its binding activity. Following cell surface biotinylation, ASGPR was immunoprecipitated, and the amounts of total and biotinylated receptor were quantified. Western blot analysis of the immunoprecipitate showed that treatment with rapamycin had no effect on the level of total receptor (Fig. 5). There was significantly less receptor on the surface of rapamycin-treated HuH7 cells than on nontreated cells. Consistent with the binding assay (Fig. 3), the antibiotic had no effect on the level of surface receptors in Trf1 cells (Fig. 5). Rapamycin-induced reduction in the plasma membrane ASGPR content of HuH-7 cells was confirmed by immunofluorescent microscopic localization of receptor on nonpermeabilized cells (Fig. 6). In agreement with the biochemical data, treatment of HuH-7 cells markedly reduced immunodetectable plasma membrane-associated ASGPR.

**Rapamycin Disrupts Sorting Heterocomplex**—To determine whether the reduction of cell surface ASGPR by rapamycin

![Graph showing the reduction of Surface ASGPR expression by Rapamycin](image-url)

**Graph**: The graph shows the reduction of Surface ASGPR expression by Rapamycin. The x-axis represents the concentration of Rapamycin (µM) and the y-axis represents the ASOR bound (% of control). The bars indicate the mean ± S.D. of four independent experiments. The results indicate that the reduction of cell surface ASGPR activity induced by rapamycin was dose-dependent.
treatment was coincident with disruption of the sorting complex, the lystate of rapamycin treated cells was passed through a GST-phosphorylated ASGPR-CD column. As an alternative approach, cells with or without prior rapamycin treatment were lysed with CHAPS to retain protein-protein interactions, and the receptor and any associated proteins were co-immunoadsorbed to covalently bound anti-ASGPR. Proteins recovered by either protocol were resolved on 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane. The membranes were probed with antibodies to HSP90 and HSP70 (Fig. 7). These results indicate that whereas total HSP70 and HSP90 expression is unaffected by rapamycin treatment, the association of HSPs 70 and 90 with GST-phosphorylated ASGPR-CD was markedly reduced by antibiotic treatment (Fig. 7A). Immunoprecipitation of ASGPR from rapamycin-treated HuH-7 cells showed a dramatic reduction in receptor-associated HSP70 and HSP90 without affecting receptor expression (Fig. 7B). The addition of a large excess of GST-phosphorylated ASGPR-CD to the lysis buffer did not alter the recovery of HSP associated with ASGPR recovered from nontreated cells, suggesting that the initial interaction had occurred in vivo (data not shown). Consistent with a potential role for the HSP heterocomplex in the trafficking of ASGPR, immunoprecipitation of the receptor from Trf1 cells did not result in the co-precipitation of HSP70 and a markedly reduced level of HSP90, independent of rapamycin treatment (Fig. 7B). To eliminate the possibility that the FKBP-rapamycin complex, which inhibits the kinase activator target of rapamycin (TOR) (15), might have a downstream effect on CK2α′′ activity, the receptor was immunoprecipitated from antibiotic-treated cells, and the level of phosphorylation was determined. As shown in figure 8, the rapamycin-induced mimicry of the trf1 mutation is not caused by a reduction of receptor phosphorylation.

DISCUSSION

In our previous reports, a membrane protein trafficking mutant (Trf1) derived from the human hepatoma cell line HuH7 was shown to be defective in the distribution of subpopulations of cell surface receptors for asialo-orsomucoid, transferrin, and mannose-terminating glycoproteins (3). The gene that complemented Trf1 cells was shown to be a novel α-subunit of CK2 designated CK2α′′ (5). It is 91% identical in amino acid se-

![Fig. 4](image1.png)

**Fig. 4. Trf1 cells do not respond to rapamycin.** HuH-7 and Trf1 cells were treated with rapamycin (1 μM) for increasing times. Cell surface ASGPR activity was determined by 125I-ASOR binding as described under "Experimental Procedures." The values shown are the means ± S.D of three independent experiments. The reduction of cell surface ASGPR expression induced by rapamycin was time-dependent and limited to the HuH-7 cell line.

![Fig. 5](image2.png)

**Fig. 5. ASGPR expression in rapamycin cells.** Confluent HuH-7 or Trf1 monolayers were treated with rapamycin (1 μM) dissolved in Me2SO or Me2SO alone for 24 h. Equivalent amounts of cell protein (50 μg) were resolved by SDS-PAGE and transferred to nitrocellulose. The membranes were probed with the polyclonal antibody against human ASGPR, and the signal was visualized by chemiluminescence after incubation with HRP-conjugated anti-rabbit secondary antibody. Total receptor, as determined by Western blot, was unaffected by rapamycin. The cell surface proteins of HuH-7 and Trf1 cells treated or not treated with rapamycin (1 μM for 24 h) were biotinylated as described under "Experimental Procedures." ASGPR was immunoprecipitated from equal amounts of cell lysate proteins, resolved on 10% SDS-PAGE and transferred for Western blot analysis. Cell surface receptor was detected by the presence of biotin with avidin-HRP. Typical results from three independent experiments are shown.

![Fig. 6](image3.png)

**Fig. 6. Reduction of surface ASGPR expression in response to rapamycin.** Following rapamycin treatment (1 μM) for 24 h, the cell surface density of ASGPR was evaluated by immunofluorescence. Fixed nonpermeabilized HuH-7 cells were incubated with anti-ASGPR antibody (1:200) and the deposition of antibody detected with fluorescein isothiocyanate-labeled goat anti-rabbit (1:100). Images were acquired on an Olympus IX70 fluorescent microscope equipped with a CCD digital camera. Control experiments without primary antibody incubation showed no signal. The figure is a representative field from two independent sets of experiments.

![Fig. 7](image4.png)

**Fig. 7. Dissociation of sorting complex by rapamycin.** A, equal amounts of cell lysates proteins from confluent HuH-7 cells (2 × 106/60-mm dish) treated or not treated with rapamycin (1 μM for 24 h) were either resolved directly on 10% SDS-PAGE or passed through a phosphorylated ASGPR-CD affinity matrix after which the washed and eluted proteins were resolved on SDS-PAGE. B, alternatively, receptor-associated proteins were co-immunoadsorbed from the lysate with anti-ASGPR covalently fixed to Sepharose-protein G prior to SDS-PAGE resolution. The recovery of protein in each fraction was determined by immunoblot analysis as described in the legend for Fig. 1. The data presented are a composite of 3–5 independent experiments.
sequence and 65.2% identical in nucleotide sequence to the previously described CK2α (9). CK2 is a ubiquitously expressed eukaryotic Ser/Thr protein kinase present in the nucleus and cytoplasm (6). Interestingly, likely physiological targets for CK2 include a number of cytoplasmic proteins that regulate intracellular trafficking (16) and elements of the microtubular network (17, 18). A comparison of the phosphorylation status of ASGPR in Trf1 and HuH7 cells indicated that the trf1 mutation results in ASGPR hypophosphorylation (5). Our identification of serine ASGPR as the major phosphorymo acid (10) and the presence of a CK2 phosphorylation motif (SSEEND) in the cytoplasmic domain of the human ASGPR are consistent with a direct role for CK2 in trafficking of this receptor.

Kinase and phosphatase activities have been shown to control both general and cargo-specific trafficking (19). In particular, CK2 phosphorylates proteins that traffic between the Golgi and the plasma membrane. For example, CK2-mediated phosphorylation of the cytoplasmic tail of the proprotein convertase, furin, is required for its localization to the trans-Golgi network (TGN) (10, 20, 21) and its recycling from early endosomes to the cell surface (22). A CK2 phosphorylation site (ESEER) on the cytoplasmic domain of the cation-dependent mannose receptor and furin has been shown to determine the high affinity interaction of the γ-subunit of the clathrin adaptor protein complex AP-1 with the mannose receptor 47-kDa tail-interactive protein (TIP47) and the phosphofurin acidic cluster-sorting protein (PACS-1). It is proposed that these interactions mediate the retrieval of these membrane proteins to the TGN (23, 24) and their recycling to the cell surface (22). Similar acidic clusters have been shown to direct several distinct sorting steps in the TGN/endocytic pathway, including localization to the TGN of carboxypeptidase D and varicellar zoster virus (25–28), basolateral sorting of low-density lipoprotein receptor and furin (29, 30), and delivery of lysosomal hydrolases by both the cation-dependent and -independent mannose-6-phosphate receptors (23, 31, 32). Only in the case of furin (PACS-1) (23) and mannose protein receptor (TIP47) (33) has the linker proteins been defined. Interestingly, early endosomal localization of internalized transferrin was unaffected by PACS-1 depletion (22), although it was shown that internalization of the transferrin receptor requires CK2 or a CK2-like kinase, suggesting the presence of a yet undefined CK2-dependent retrieval protein or proteins.

Utilizing the phosphorylated ASGPR-CD as bait, we isolated a heterocomplex of potential sorting proteins comprising members of both the heat shock and immunophilin families. The major heat shock proteins, HSP90 and HSP70, were well recognized molecular chaperones that are required for the proper folding and trafficking of many proteins involved in signal transduction (34, 35). Recently, gp96, HSP90, and HSP70 were shown to selectively associate with tumor-specific cytotoxic T lymphocyte epitopes, implying that this combination of HSP can act in distinct intracellular compartments to traffic antigenic peptides to major histocompatibility complex (MHC-I) molecules (36). 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 (37). Such proteins include members of the immunophilin family, such as the recovered FKBP and cyclosporin A-binding protein. In as much as immunophilins have been shown to exist in independent heat shock protein heterocomplexes (38), the recovered proteins that are associated with the ASGPR-CD may reflect a mixture of potential sorting heterocomplexes, each with a different temporal function regulating ASGPR trafficking.

Treatment of parental HuH-7 cells with the macrolide antibiotic rapamycin mimicked the Trf1 phenotype by decreasing cell surface ASGPR by ~50% without affecting total receptor expression or phosphorylation. Trf1 cells exhibit a pleiotropic phenotype that alters not only ASGPR and transferrin receptor expression but also the trafficking of additional unrelated plasma membrane proteins (4). The trf1 mutation results in an ~50% reduction in the cell surface expression of susceptible proteins at the plasma membrane. Early studies established the functional (39) and metabolic (40) separation of the cell surface ASGPR population from that of the larger intracellular receptor pool. This concept of two subsets of surface receptors, designated States 1 and 2 by Weigel and Oka (41), has continued to gained support. Although the biochemical basis for these two types of receptor has not been established, changes in phosphorylation (42) and acylation (43) were suggested to affect the transition from State 1 to State 2. Only State 2 receptors are proposed to be susceptible to modulation by a variety of cell perturbants, including reduced temperature, colchicine, cytokines, phorbol esters, monensin and chloroquine, azide, vanadate, or ethanol (44). In all cases, these agents cause an approximate 50% reduction in cell surface ASGPR activity, and in some cases, a redistribution of cell surface receptors was confirmed as the cause for this reduction. The selective reduction of cell surface ASGPR in Trf1 cells also appeared to be due to the redistribution of State 2 receptors to an internal pool (3). The failure of the Trf1 cell line to respond to rapamycin (Fig. 4) indicates that the distribution of State 1 receptors is not responsive to the antibiotic. This notion is consistent with the newly described H2c isoform of the receptor (45), which lacks a CK2 phosphorylation motif. As suggested by the authors, State 2 receptors would contain H1-H2b and would be responsive to CK2α+ phosphorylation, whereas State 1 receptors, containing H1-H2c, would not be affected by the antibiotic.

Analysis of the ASGPR-CD heterocomplex following rapamycin treatment indicated that the antibiotic prevented the formation or disrupted the association of HSP with the ASGPR-CD (Fig. 6). In a somewhat analogous study, evidence was presented that the cytosolic HSP-immunophilin chaperone complex of caveolin, when disrupted by rapamycin, prevents rapid cholesterol transport to the plasma membrane (14). Although it has been proposed that the rapid insertion of caveolin into the plasma membrane is microtubule-based, the question of whether the inhibition of caveolin trafficking by rapamycin was due to the disruption of microtubular interaction was not resolved (11). In a recent study, it was shown that dynin, a molecular motor for minus end movement along microtubules, was present in glucocorticoid receptor HSP90 heterocomplexes,
and it was suggested that FKBP provided the molecular link between the receptor heterocomplex and cytoplasmic dynein (46). Trafficking of ASGPR containing vesicles along the microtubular network is well established (47), and the dynamic interaction of its heat shock protein-immunophilin complex with specific molecular motors could provide the direction necessary to maintain the steady state distribution of the receptor.

Based on these results, we propose that the interaction of a heterocomplex containing a combination of gp96, HSP90, HSP70, FKBP-18, and cyclophilin-A is dependent upon phosphorylation of ASGPR-CD by CK2α’ (48) and that this differential phosphorylation establishes the distribution of ASGPR between the plasma membrane and the endosomal pool.

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