The EMBO Journal vol.8 no.10 pp.2855-2861, 1989

Endocytosis and recycling of subunit H1 of the asialoglycoprotein receptor is independent of oligomerization with H2

Iris Geffen, Hans Peter Wessels, Jürgen Roth[1], Michael A.Shia[2] and Martin Spiess

Department of Biochemistry and [1]Interdepartmental Electron Microscopy, Biocenter, University of Basel, CH-4056 Basel, Switzerland and [2]Whitehead Institute for Biomedical Research, Nine Cambridge Center, Cambridge, MA 021412, USA

Communicated by G.Semenza

The human asialoglycoprotein receptor is composed of two homologous subunits, H1 and H2. By expressing the two subunits in transfected fibroblast cell lines, it has been shown previously that the formation of a hetero-oligomeric complex is necessary for the transport of H2 to the plasma membrane and for high-affinity ligand binding. Here we show that subunit H1, when expressed in the absence of H2, is capable of internalization through coated pits and recycling. The kinetics of these processes are very similar to those of the H1-H2 complex. To study endocytosis in the absence of ligand binding, the cell surface was labeled at 4°C with the ^{125}I-iodinated impermeant reagent sulfosuccinimidyl-3-(4-hydroxyphenyl) propionate, the cells were incubated at 37°C for different times and the amount of internalized receptor was determined by protease digestion of surface proteins and immunoprecipitation. Similarly, recycling of surface-labeled and then internalized receptor protein was studied by monitoring its reappearance on the surface in the presence of exogenous protease. Our results show that subunit H1 contains all the signals necessary for receptor endocytosis and recycling independent of ligand binding.

Key words: asialoglycoprotein receptor/cell surface labeling/endocytosis/recycling

Introduction

The asialoglycoprotein (ASGP) receptor is a constituent of the plasma membrane of hepatocytes that specifically binds terminal glactose and *N*-acetylgalactosamine residues on serum glycoproteins (reviewed by Ashwell and Harford, 1982; Breitfeld *et al.*, 1985). Following binding, the ligand is endocytosed into clathrin-coated vesicles and delivered to an acidic endosomal compartment where the ligand-receptor complex dissociates. While the ligand is transported to lysosomes for degradation, the receptor recycles back to the plasma membrane. By this process of receptor-mediated endocytosis, ASGPs are rapidly cleared from the blood circulation.

Analysis of the isolated receptor proteins and of cloned cDNAs revealed that there are two genes encoding homologous proteins, H1 and H2 in the human system (Spiess and Lodish, 1985; Bischoff and Lodish, 1987), and RHL-1 (rat hepatic lectin) and RHL-2/3 (two differently glycosylated products of the second gene) in rat hepatocytes (Drickamer *et al.*, 1984; Halberg *et al.*, 1987). H1 and RHL-1 are expressed to a 3- to 6-fold higher extent than their respective homologs. The existence and evolutionary conservation of two homologous galactose-binding proteins raised the question about their function either as separate receptors or as constituents of a single multicomponent receptor. McPhaul and Berg (1986) found that HTC cells acquired the ability to internalize and accumulate fluorescent labeled asialoorosomucoid only when transfected with the cDNAs of both rat proteins RHL-1 and RHL-2, suggesting that the two proteins do not constitute separate entities, but that they must interact with one another to yield a functional receptor. This notion was confirmed by the finding that in non-ionic detergent a complex of the three rat polypeptides is immunoprecipitated by antibodies specific either to RHL-1 or RHL-2/3 (Sawyer *et al.*, 1988). Similarly, Bischoff *et al.* (1988) showed that in the human hepatoma cell line HepG2, antibody-induced degradation of the ASGP receptor using either anti-H1 or anti-H2 antiserum resulted in a simultaneous loss of both polypeptides.

The clearest evidence for the ASGP receptor being a hetero-oligomeric complex of H1 and H2 was provided by Shia and Lodish (1989) who analyzed the fate and properties of either polypeptide when expressed separately or together in stably transfected fibroblasts. In the absence of H2, H1 is transported to the cell surface normally, but does not bind ligand. In contrast, H2 expressed alone is rapidly degraded and does not reach the plasma membrane. In cells expressing both proteins, H2 is 'rescued' to the cell surface, and ligand binding and uptake is observed as in HepG2 cells. An association of the two polypeptides is thus necessary for the transport of H2 to the plasma membrane. Since the individual

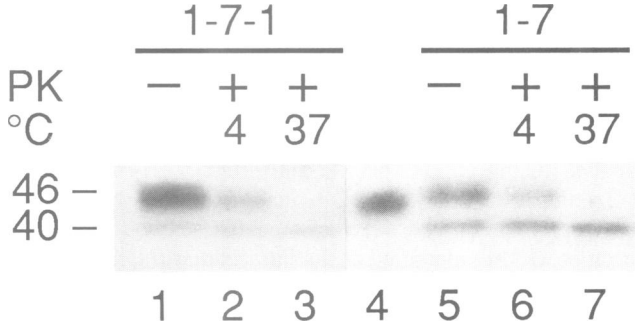

Fig. 1. Cellular distribution of the ASGP receptor in 1-7-1 and 1-7 cells. The distribution of the ASGP receptor proteins between the cell surface and intracellular compartments in 1-7-1 and 1-7 cells was determined by immunoblot analysis (as described in Materials and methods). Receptor protein of control cells (**lanes 1** and **5**), of cells digested with proteinase K (PK) for 30 min at 4°C (**lanes 2** and **6**) and at 37°C (**lanes 3** and **7**) are shown. In **lane 4**, the ASGP receptor purified from human liver was analyzed for comparison. The positions of the 40 kd high-mannose precursor form and the 46 kd complex-glycosylated mature form of the receptor are indicated.

polypeptides independently bind to immobilized ASGPs or galactose, these results also show that the formation of a complex of H1 and H2 is essential for high-affinity ligand binding, most probably by creating a co-operative arrangement of the binding sites of the subunits.

Although the ASGP receptor is the only endocytic receptor known composed of two non-identical subunits (except for the insulin receptor whose α and β chains, however, are produced by processing of a single precursor protein), many other receptors have been shown to form dimers or oligomers of identical polypeptides (e.g. the receptors for transferrin and mannose-6-phosphate; Schneider *et al.*, 1981; Kornfeld, 1987). It is therefore conceivable that for the formation of a receptor complex, the presentation of multiple (possibly co-operative) recognition domains is a prerequisite for the clustering in coated pits and thus endocytosis. In this study, we have investigated whether the association of H1 and H2 is also necessary for the process of endocytosis and recycling of the ASGP receptor, i.e. whether subunit H1 can be endocytosed in the absence of H2.

Results

We have analyzed cell surface distribution, endocytosis and recycling of the ASGP receptor polypeptides in the two stable cell lines, 1-7 and 1-7-1, which are derived from NIH3T3 fibroblasts by introducing receptor cDNA in a retroviral expression vector (Shia and Lodish, 1989). 1-7 cells express only subunit H1 of the ASGP receptor: ligand binding cannot be detected although H1 is efficiently transported to the plasma membrane. 1-7-1 cells are derived from 1-7 cells and express both receptor subunits H1 and H2 which form a functional ASGP receptor capable of ligand binding and uptake with characteristics very similar to those of the receptor in HepG2 hepatoma cells (Shia and Lodish, 1989).

A first indication of whether subunit H1 is capable of internalization and recycling in the absence of H2 was obtained by analyzing its distribtuion between the plasma membrane and intracellualar compartments. The cells were incubated with proteinase K for 30 min either at 4 or 37°C to digest the receptor on the cell surface. At 4°C, membrane traffic is arrested and only receptor exposed on the plasma membrane is digested. At 37°C, all functional receptor engaged in endocytosis and recycling is expected to appear at the cell surface within the incubation time and to be degraded. After digestion, receptor that was not susceptible to proteolysis, i.e. did not appear at the surface, was determined by SDS gel electrophoresis and immunoblot analysis using an H1-specific antiserum.

In untreated cells (Figure 1, lanes 1 and 5), two forms of H1 of 40 and 46 kd were detected. These two forms have been previously identified as the intracellular, high-mannose glycosylated precursor and the complex glycosylated mature form respectively (Schwartz and Rup, 1983; Shia and Lodish, 1989). The latter migrates with an electrophoretic mobility very similar to that of the ASGP receptor purified from human liver (Figure 1, lane 4). After protease digestion at 4°C, ~40% of mature H1 in 1-7-1 cells was protected from protease digestion (lane 2), thus constituting the fraction of the receptor located intracellularly. Surface shaving of 1-7-1 cells at 37°C resulted in complete disappearance of mature H1 (lane 3), consistent with the notion that H1 as part of a functional ASGP receptor complex is continuously

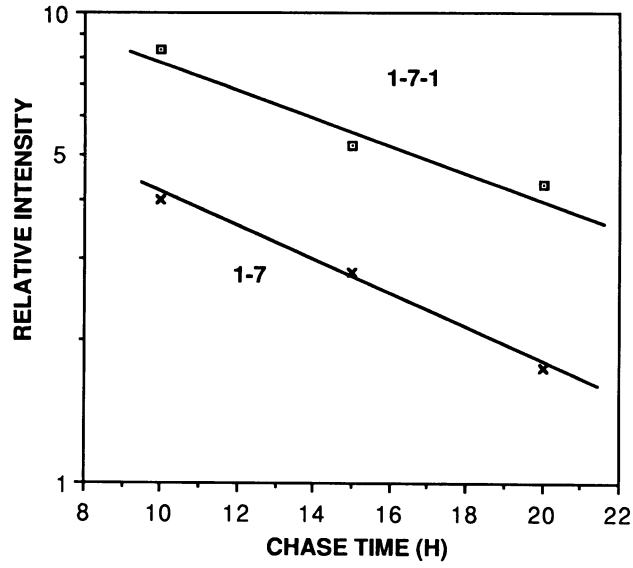

Fig. 2. Half-life of mature ASGP receptor in 1-7 and 1-7-1 cells. Cells were pulse-labeled for 30 min with [^{35}S]methionine and [^{35}S]cysteine and then chased with normal growth medium for 10–20 h (after 10 h the high-mannose glycosylated precursor form had disappeared). The receptor was immunoprecipitated and analyzed by gel electrophoresis and fluorography. Upon densitometric scanning of duplicate experiments the half-life was determined to be ~8 h in 1-7 cells and ~11 h in 1-7-1 cells.

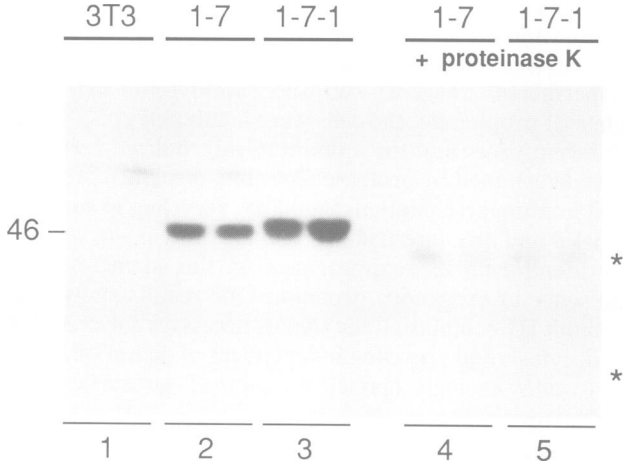

Fig. 3. Labeling and digestion of cell surface ASGP receptor. 3T3, 1-7, and 1-7-1 cells, as indicated, were surface labeled with [^{125}I]sulfo-SHPP at 4°C. The cells were lysed either immediately (**lanes 1–3**) or after proteinase K digestion of the cell surface at 4°C (**lanes 4** and **5**), and ASGP receptor was immunoprecipitated and analyzed by SDS–gel electrophoresis and autoradiography. The position of the 46-kd complex-glycosylated ASGP receptor is indicated. The asterisks indicate the position of two receptor fragments which are precipitated by the antibody.

recycled to the plasma membrane and endocytosed at a rate that exposes the entire population to the protease on the cell surface within 30 min. The intracellular precursor of 40 kd remained unaffected by digestion of the cell surface at either temperature, indicating that the cells stayed intact during the experiment. Using the same method, 60% of the mature form of the naturally expressed ASGP receptor in HepG2 cells was found on the cell surface (Wessels *et al.*, 1989) and 100% was digestible on the cell surface within 30 min at 37°C (not shown).

In 1-7 cells, ~50% of total mature H1 is exposed on the cell surface and therefore was degraded when the cells were incubated with proteinase K at 4°C (lane 6). As in 1-7-1 and HepG2 cells, all of the 46 kd receptor of 1-7 cells was digested within 30 min at 37°C (lane 7), indicating that also in the absence of H2 the intracellular pool of H1 is rapidly transported to the cell surface. The half-life of the receptor is much longer than the time required for all receptors to appear on the plasma membrane: by metabolic labeling the half-life was determined to be ~8 h and ~11 h in 1-7 and 1-7-1 cells respectively (Figure 2). The high rate of exocytosis of H1 to the cell surface therefore suggests that in untreated cells the surface population of H1 must be internalized at approximately the same rate.

In order to study the behavior of H1 in 1-7 cells, and because these cells do not bind ASGPs, we devised a protocol to monitor directly endocytosis of receptor polypeptides in the absence of ligand. The cell surface is first labeled at 4°C by incubation with ^{125}I-iodinated sulfosuccinimidyl-3-(4-hydroxyphenyl) propionate (sulfo-SHPP), a water-soluble, and thus impermeant, amino group-specific reagent (Thompson *et al.*, 1987). The labeled receptor was analyzed by immunoprecipitation using an antiserum raised against purified receptor, SDS–gel electrophoresis and autoradiography. As is shown in Figure 3, the immunoprecipitated material was specific for cells expressing receptor (lanes 2 and 3) and was absent from control 3T3 fibroblasts (lanes 1). Only the mature form of the receptor was labeled, confirming that the modification is restricted to the cell surface. If the cells were kept at 4°C, the radioactive receptor could be completely digested with proteinase K on the cell surface (lanes 4 and 5); only small amounts of two degradation products could be detected (indicated by asterisks). To study endocytosis, the surface-labeled cells were incubated at 37°C for different times after which the cells were rapidly cooled and the receptor which was still exposed on the surface was digested by protease at 4°C. Receptor that had been internalized during the 37°C chase and thereby had acquired resistance to exogenous protease was then immunoprecipitated and quantitated (Figures 4 and 5). In control experiments we determined that the ligand-binding properties of 1-7-1 cells were unaffected by the surface modification (not shown). The iodinated receptor both of 1-7 and 1-7-1 cells could be cross-linked with difluorodinitrobenzene to dimers and trimers (data not shown) like the unlabeled receptor (Shia and Lodish, 1989), suggesting that the modification did not impair its ability to form oligomers. In addition, after 4 h at 37°C ~60% of the labeled receptor protein in 1-7 cells were recovered and >90% in 1-7-1 cells. The corresponding half-lives of surface-iodinated H1 of ~6 h in 1-7 cells and >10 h in 1-7-1 cells are in agreement with the half-lives of ~8 h and ~11 h respectively, determined for biosynthetic labeled mature receptor in the two cell lines (see above, Figure 2). These findings suggest that the behavior of the labeled receptor protein is not detectably altered by the modification, and that the turnover of the receptor proteins (i.e. biosynthesis and endogenous degradation) is negligible within the time of the experiments.

The results of the endocytosis assay for 1-7-1 cells are shown in Figure 4 (lanes 1–5). Following incubation at 37°C, increasing amounts of the surface-labeled receptor were protected from digestion. Densitometric scanning of

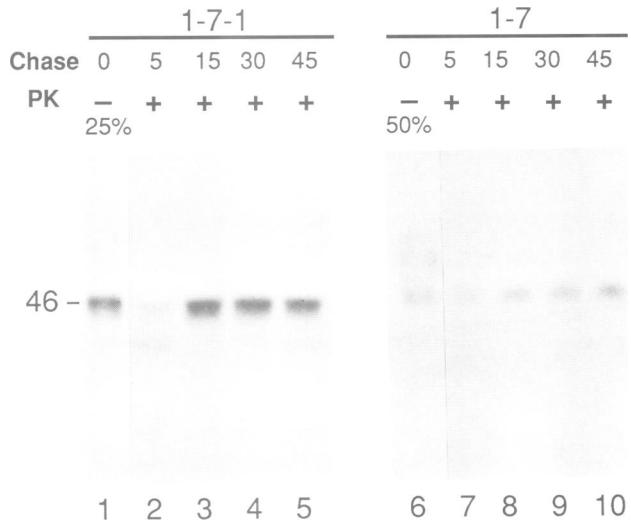

Fig. 4. Endocytosis of surface-labeled ASGP receptor in 1-7-1 and 1-7 cells. The surface of 1-7-1 and 1-7 cells was labeled with [^{125}I]sulfo-SHPP at 4°C. The cells were incubated at 37°C for increasing chase periods (as indicated in minutes), after which the cells were chilled and surface proteins digested with proteinase K at 4°C. The cells were then lysed and the ASGP receptor was immunoprecipitated and analyzed by SDS–gel electrophoresis and autoradiography (**lanes 2–5** and **7–10**). Of control 1-7-1 and 1-7 cells kept at 4°C and not digested with protease aliquots of 25 and 50% respectively were analyzed in parallel (**lanes 1** and **6**).

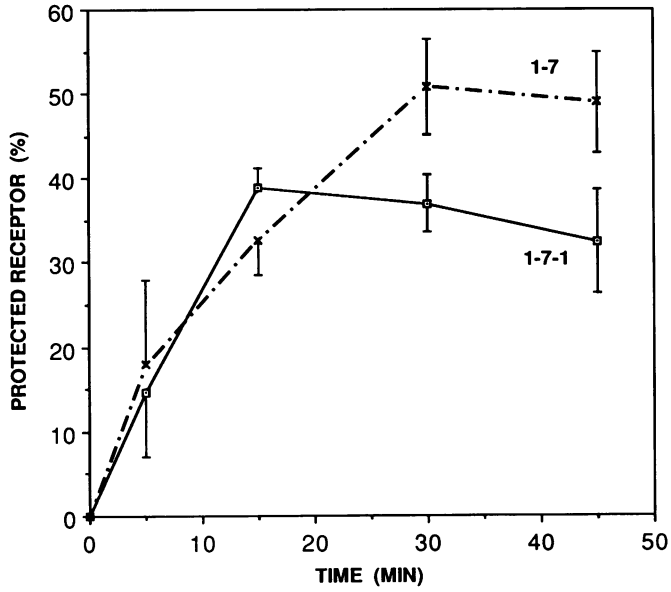

Fig. 5. Time course of endocytosis of ASGP receptor in 1-7-1 and 1-7 cells. Endocytosis assays as shown in Figure 4 were quantitated by densitometric scanning of the autoradiographs. The average values of triplicate samples (including those shown in Figure 4) are plotted and the standard deviations indicated.

the protected fractions from three independent determinations showed that almost 40% was inaccessible to protease after 15 min (Figure 5), indicating that endocytosis took place. After longer incubation, the protected fraction leveled off, most likely due to mixing of the labeled receptor on the cell surface with initially intracellular, unlabeled protein transported to the plasma membrane.

The result of the same experiment performed with 1-7 cells is shown in Figure 4 (lanes 6–10) and Figure 5 and indicates

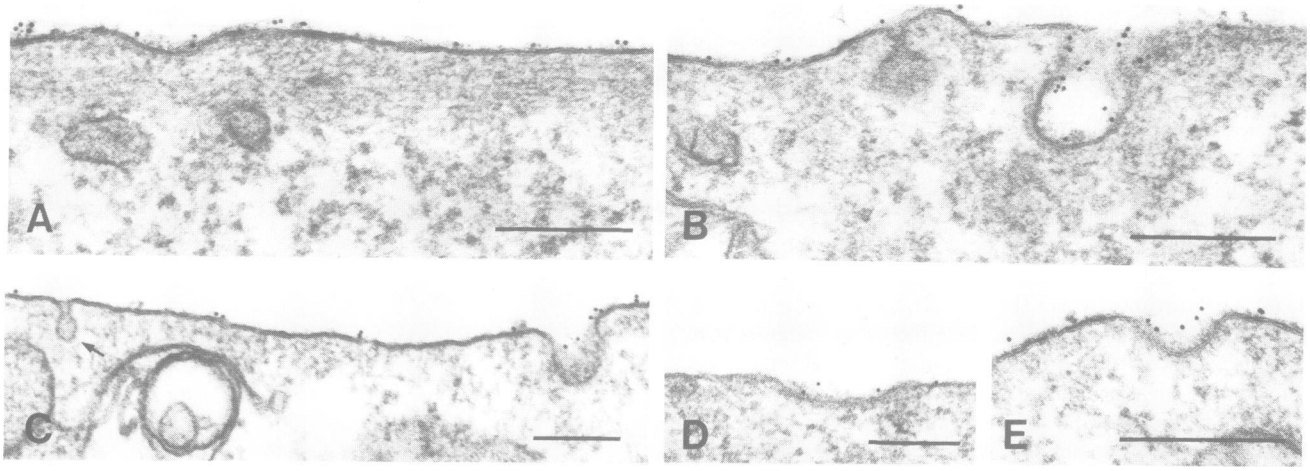

Fig. 6. Immunolocalization of the ASGP receptor in the plasma membrane of 1-7-1 and 1-7 cells. The cell surface distribution of the ASGP receptor was revealed by pre-embedding immunolabeling. In 1-7-1 cells, gold particle label indicative of immunoreactivity for the ASGP receptor is found along the plasma membrane as well as in coated pits (**A**) and coated membrane invaginations (**B**). The same gold particle distribution is observed in 1-7 cells. Note the presence of labeling in coated membrane invaginations (**C,E**) and coated pits (**D**) but absence in a smooth membrane invagination (arrow in **C**). In 3T3 cells, which served as a control, no plasma membrane labeling was detectable (not shown). Bars: 0.25 μm.

that the behavior of H1 in the absence of the second subunit is very similar to that of the functional receptor in 1-7-1 cells. Approximately half of the receptor labeled on the cell surface at time zero acquired resistance to exogenous protease within 30 min at 37°C, indicating that subunit H1 is endocytosed also when it is not associated with H2. The apparent rate of endocytosis (compare the early time points) was very similar in the two cell lines. The final values of resistant radiolabeled receptor corresponds closely to the intracellular fraction of the receptor of ~40% in 1-7-1 and ~50% in 1-7 cells (as determined by the immunoblot analysis shown in Figure 1), suggesting that the labeled and the unlabeled receptor have essentially equilibrated within ~30 min.

Independently, the distribution of receptor in the plasma membrane and in coated pits was analyzed and compared in 1-7-1 and 1-7 cells by immunoelectron microscopy. The cells were labeled *in situ* using a specific antiserum and protein A–gold complexes. The plasma membrane distribution of the receptor was the same in 1-7-1 and 1-7 cells, except that the labeling intensity was higher in 1-7-1 cells. As is shown in Figure 6, gold particles were found along the smooth plasma membrane regions and microvilli as well as in coated pits and coated membrane invaginations.

Mixing of the labeled receptor pool initially on the surface and the unlabeled, initially intracellular pool (as suggested by the experiments described above in Figures 4 and 5) implies recycling of intracellular receptors to the plasma membrane. To monitor directly this process, cells were surface labeled on ice as in the previous experiment and endocytosis was allowed to take place in a 20 min incubation at 37°C. This period was chosen because then the endocytosed fraction of labeled receptor was close to maximal in both cell lines. The cells were then rinsed and proteinase K was added for increasing times at 37°C. Within <2 min all the receptor proteins on the surface were digested (this has been determined with control cells that were protease treated at 37°C immediately after labeling; data not shown). During further incubation, recycled receptor molecules were digested immediately as they appeared at the plasma membrane. As is shown in Figure 7, the receptor protein that had been labeled at the cell surface at time zero (lanes

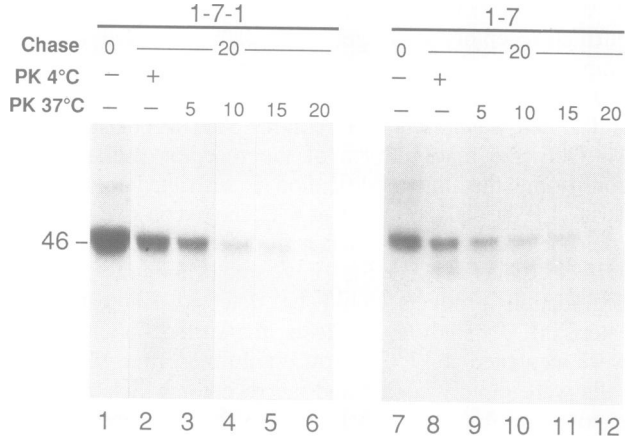

Fig. 7. Recycling of internalized ASGP receptor in 1-7-1 and 1-7 cells. The surface of 1-7-1 and 1-7 cells was labeled with [^{125}I]sulfo-SHPP at 4°C (**lanes 1** and **7**). Endocytosis was allowed to take place at 37°C for 20 min (chase). To determine the amount of receptor internalized, cells were surface shaven with proteinase K at 4°C (**lanes 2** and **8**). To monitor recycling, cells were incubated at 37°C with proteinase K for the different times (indicated in minutes; **lanes 3–6** and **9–12**). Of all samples, ASGP receptor was immunoprecipitated and analyzed by SDS–gel electrophoresis and autoradiography.

1 and 7) and that had been endocytosed into a protease-protected compartment within 20 min (lanes 2 and 8) reappeared at the cell surface and was digested by exogenous protease within ~20 more minutes (lanes 3–6 and 9–2). Quantitation of autoradiograms from three independent determinations is shown in Figure 8 (after 20 min the amount of protein recovered was too small to be accurately quantitated and was not indicated). The pattern of receptor return to the plasma membrane is similar in the two cell lines, the apparent rate of exocytosis being slightly lower in 1-7 cells. This might account for the somewhat smaller fraction of mature receptor present on the cell surface in this cell line.

To exclude the possibility that the loss of intracellular receptor was due to internalization of protease during the 37°C incubation rather than to recycling of endocytosed receptor, a similar experiment was performed in which the protease incubations were done at 4°C (see Materials and

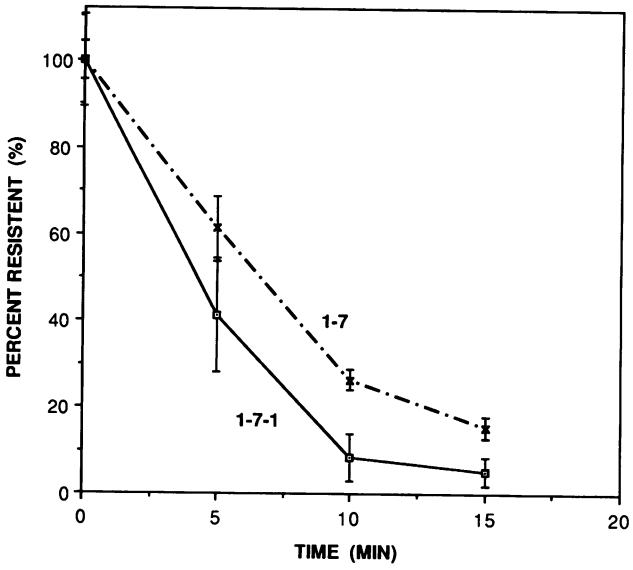

Fig. 8. Time course of recycling internalized receptor. Recycling assays as shown in Figure 7 were quantitated by densitometric scanning of the autoradiographs. The average values of triplicate samples (including those shown in Figure 7) are plotted and the standard deviations indicated. At the last time point of incubation (20 min), the amount of residual receptor is too low in both cell lines to be accurately quantitated.

methods). After 5 min chase at 37°C, 49 and 36% of previously internalized labeled receptor in 1-7 and 1-7-1 cells respectively were still protected from exogenous protease; only 29 and 26% respectively were intracellular after 10 min. These results confirm that endocytosed subunit H1, whether H2 is expressed or not, is rapidly recycled to the cell surface. The values obtained are very similar to those determined with protease continuously present at 37°C (Figure 8), suggesting that fluid-phase uptake of protease does not contribute significantly to the loss of receptor. Using the second method, however, recycled molecules can escape digestion by reinternalization, especially during longer chase times.

Discussion

The ASGP receptor belongs to a group of transport receptors that carry their ligands from the cell surface into the cell for degradation and themselves return to the plasma membrane (reviewed by Goldstein *et al.*, 1985). These receptors were shown to continuously enter the cell and recycle back to the surface independently of binding of ligand. Evidence for such constitutive endocytosis of the ASGP receptor was obtained by electron microscopy which showed clustering of receptor in coated pits of rat hepatocytes also in the absence of ligand (Wall and Hubbard, 1981). Furthermore, when HepG2 cells were treated with lysosomotropic agents, which are presumed to trap receptors in endosomal compartments, the ASGP receptor was found to be depleted from the plasma membrane with or without ASGPs added (Schwartz *et al.*, 1984).

The procedure used in this work to monitor receptor-mediated endocytosis (trace-labeling of the cell surface and assay for receptors that have acquired resistance to exogenous protease) lends itself excellently for the analysis and quantitation of constitutive endocytosis and is applicable also to other receptor systems. This method does not involve toxic substances (such as lysosomotropic agents) that could non-specifically affect the processes to be studied. Labeling by iodinated sulfo-SHPP is restricted entirely to the cell surface, as is demonstrated by the fact that the intracellular precursor form of the ASGP receptor is not labeled. The modification is performed at an extremely low stoichiometry that is very unlikely to affect cellular processes. However, since only the labeled subpopulation of the protein under investigation is monitored in the experiment it is important to analyze the stability of the modifed molecules in comparsion to that of the total population in unlabeled cells. The half-life time of the ASGP receptor was found not be affected by the modification, indicating that the labeled receptor most likely behaves like the unmodified one.

Two reagents (DPSgt and DPSgtc) very similar to the one used in this study have been developed very recently by Bretscher and Lutter (1988). In these reagents the reactive sulfosuccinimidyl group is coupled by a disulfide linkage to an oligopeptide containing a tyrosine residue that can be iodinated. These molecules can be applied for experiments like the ones described here. Since the label exposed on the cell surface can be removed by impermeant reducing agents, they are especially useful to study proteins not readily digestible by proteases.

The main finding of this work is that the formation of a complex between subunits H1 and H2, although essential for transport and maturation of H2 and for high-affinity ligand binding, is not necessary for endocytosis and recylcing: H1 is endocytosed and recycled also in the absence of H2. As judged by immunoelectron microscopy, H1 alone (in 1-7 cells) exhibits the same cell surface distribution as do H1−H2 complexes (in 1-7-1 cells) and is equally found in coated pits and coated membrane invaginations. Moreover, the rates observed in cells that express H1 alone (1-7) or both subunits together (1-7-1) are very similar. The difference detected in the rate of recycling of internalized receptor protein back to the cell surface, which is somewhat lower in cells expressing H1 alone, could account for the higher percentage of mature protein found on the surface of 1-7 cells (50% versus 40% in 1-7-1 cells).

Since most of the endocytic receptors were shown to form covalent (e.g. transferrin receptor and insulin receptor) or non-covalent (e.g. low-density lipoprotein receptor, mannose-6-phosphate receptor) dimers or oligomers, it is conceivable that complex formation is essential for the mechanism of endocytosis. In particular, the first step of the process, clustering in coated pits, might depend on multiple interactions of the receptor complex with the postulated adaptor molecules associated with the clathrin coat (Pearse, 1987, 1988). For the human ASGP receptor, it has been shown by cross-linking studies in HepG2 cells that the two subunits H1 and H2 are associated in a ratio of 2:1 to form trimers or even higher complexes (Bischoff *et al.*, 1987). Cross-linking studies in 1-7 cells indicate that H1 when expressed alone is associated in oligomers, probably trimers. Our findings thus do not rule out a possible role of oligomerization in endocytosis.

The situation of H1 in 1-7 cells is reminiscent of the chicken hepatic lectin (CHL), which is considered the avian homolog of the mammalian ASGP receptor (Ashwell and Harford, 1982). CHL is composed of a single subunit probably forming a hexamer (Loeb and Drickamer, 1987).

Considerable homology between CHL and the ASGP receptors (H1, 26%; H2, 28%) suggests a common evolutionary origin (Drickamer *et al.*, 1984). Sequence homology, however, is confined to the carboxy-terminal carbohydrate-binding domain. The rest of the polypeptide, including a segment that has been implicated in subunit interaction in CHL (Loeb and Drickamer, 1987), was not conserved.

Clearly, subunit H1 contains all the signals necessary for endocytosis and recycling. Cell lines transfected with H1 alone or with mutants of H1 can thus be used to study the mechanisms of these processes and the signals involved. In the low-density lipoprotein receptor and other systems, the cytoplasmic domain and particularly a tyrosine residue in this domain have been implicated in recognition by clathrin-associated proteins in coated pits (Davis *et al.*, 1986, 1987; Lazarovits and Roth, 1988; Pearse, 1988). Subunit H1 contains in its cytoplasmic segment a single tyrosine at position 5 which is not conserved in H2: at the homologous position there is a phenylalanine. Whether the tyrosine residue in H1 is involved in endocytosis and the phenylalanine in H2 is also functional (as is the phenylalanine substitution in the low density lipoprotein receptor; Davis *et al.*, 1987) remains to be determined.

Materials and methods

Cell culture

The cell lines 1-7 and 1-7-1 were derived from mouse NIH3T3 fibroblasts as described by Shia and Lodish (1989). Media were purchased from Gibco Laboratories. Cells were grown in Dulbecco's minimal essential medium supplemented with 2 mM L-glutamine, 7.5% bovine calf serum (from Inotech, Switzerland), 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C with 7.5% CO_2.

Determination of receptor distribution

Cells were grown to 90% confluency in 15 mm wells, rinsed with ice-cold phosphate-buffered saline (PBS, 12.5 mM sodium phosphate, pH 7.5, 125 mM NaCl) and incubated for 30 min at 4 or 37°C with PBS containing 1 mg/ml proteinase K (Serva) and 2.5 mM EDTA. Digestion was stopped by addition of 2 mM phenylmethylsulfonyl fluoride (PMSF; Fluka) and pelleting of the cells by gentle centrifugation in a microfuge. The cells were resuspended in hot gel sample buffer (2% SDS, 80 mM Tris–HCl, pH 6.8, 0.1 M β-mercaptoethanol). Control cells were directly lysed in hot sample buffer. The samples were immediately boiled for 2 min and aliquots fractionated by 15% SDS–PAGE and transferred to nitrocellulose filters according to Towbin *et al.* (1979). The filter was then probed with a specific antibody raised against a synthetic peptide corresponding to residues 277–287 of ASGP receptor subunit H1 and affinity-purified with immoblized ASGP receptor isolated from human liver (Baenziger and Maynard, 1980), followed by incubation with [^{125}I]protein A (10^6 c.p.m./ml). Autoradiographs were quantitated by densitometric scanning using a Camac TLC scanner II equipped with an integrator.

Cell surface labeling

To label selectively surface proteins, the impermeant reagent sulfosuccinimidyl-3-(4-hydroxyphenyl) propionate (sulfo-SHPP; from Pierce) was used as described by Thompson *et al.* (1987). Sulfo-SHPP is a water-soluble, membrane-impermeant derivative of the 'Bolton–Hunter reagent' SHPP (Bolton and Hunter, 1973). For 5 ml of labeling solution, 2 μl sulfo-SHPP (0.2 mg/ml in DMSO) were iodinated by successive addition of 5 μl [^{125}I]NaI (0.1 mCi/μl; New England Nuclear), 5 μl chloramine T (5 mg/ml in 0.5 M Na-phosphate, pH 7.5; Fluka), 50 μl 4-hydroxyphenylacetic acid (1 mg/ml in H_2O.; Fluka), and 5 μl Na-disulfite (12 mg/ml in 50 mM Na-phosphate, pH 7.5; Merck). All solutions were freshly prepared before use. The reaction mixture was diluted into 5 ml of cold PBS and immediately added to the cells (1 ml per 35 mm well) which had been grown to 90% confluency. After 30 min on ice, the cells were rinsed three times with PBS containing 1 mg/ml lysine.

Biosynthetic labeling

To determine the half-life of the mature, complex-glycosylated ASGP receptor, confluent cells in 60 mm tissue culture plates were labeled for 30 min at 37°C with [^{35}S]methionine and [^{35}S]cysteine (Bischoff and Lodish, 1987). The cells were rinsed twice with PBS and then incubated with complete growth medium for 10, 15 and 20 h (after 10 h the high-mannose glycosylated precursor form had disappeared). The labeled receptor was analyzed by immunoprecipitation, gel electrophoresis and fluorography, and quantitated by densitometric scanning.

Immunoelectron microscopy

Specific labeling of plasma membrane ASGP receptor was analyzed by immunoelectron microscopy applying the protein A–gold technique (Roth *et al.*, 1978). Monolayer cultures of 1-7-1, 1-7 and NIH3T3 cells were fixed *in situ* in the dishes with 4% (para)formaldehyde in PBS for 10 min at 37°C, briefly washed with PBS, covered with 50 mM NH_4Cl in PBS for 30 min to amidinate free aldehyde groups and processed *in situ* for cell surface labeling as follows. Cell monolayers were covered with 2% defatted milk powder in PBS for 5 min and the incubated with antiserum (100-fold dilution in 1% defatted milk powder in PBS) for 45 min at room temperature. After two rinses with PBS for 2 min each, incubation with protein A–gold complexes (8 nm gold particles, diluted to an $OD_{525\ nm}$ = 1.5 with PBS containing 1% bovine serum albumin, 0.05% Triton X-100 and 0.05% Tween 20) was carried out for 30 min at room temperature, followed by two rinses with PBS for 2 min each, fixation in 2% glutaraldehyde in PBS for 2 h and fixation in reduced osmium tetroxide for 20 min. Finally, cells were mechanically removed, enclosed in 2% agar and embedded in Epon 812 according to the standard procedure. Thin sections were counterstained with 3% aqueous uranyl acetate (20 min) and lead acetate (5 min) and examined with a Zeiss EM 10 electron microscope.

Endocytosis assay

Surface-labeled cells were incubated with complete medium at 37°C. At different times, cells were rinsed with ice-cold PBS and the surface was shaved with 1 mg/ml proteinase K in PBS with 2.5 mM EDTA at 4°C for 30 min. Digestion was stopped by addition of 2 mM PMSF and gentle pelleting. The cells were lysed in lysis buffer (1% Triton X-100, 0.5% deoxycholate, 2 mM PMSF in PBS) and undigested receptor was immunoprecipitated with a rabbit polyclonal antiserum raised against isolated human ASGP receptor and with protein A–Sepharose (Pharmacia). The immunoprecipitates were dissociated in gel sample buffer by boiling for 2 min and analyzed by gel electrophoresis and autoradiography. Quantitation was performed by densitometric scanning of the autoradiographs.

Recycling assay

Following surface labeling, endocytosis was allowed to take place for 20 min at 37°C in complete medium. The cells were then incubated with proteinase K (1 mg/ml in PBS with 2.5 mM EDTA) at 37°C for different times to digest the surface receptors and the receptor reappearing at the cell surface during protease incubation. Digestion was stopped and intact receptor was immunoprecipitated as described above.

Alternatively, surface-labeled cells were incubated at 37°C for 45 min and then treated with proteinase K at 4°C for 30 min. The digestion was stopped by addition of PMSF (2 mM final concentration) and removed by gently pelleting the cells. Cells were resuspended in pre-warmed medium and incubated for 0, 5 and 10 min at 37°C after which they were chilled on ice, gently pelleted, and again treated with proteinase K at 4°C for 30 min. Interacellular radioactive receptor was analyzed as above.

Acknowledgements

We thank C.Handschin for excellent technical assistance and Dr H.F.Lodish for his support and for critically reading the manuscript. We are grateful to B.Marshallsay for her help with electron microscopy and D.Way for EM photography. This work was supported by grant 3.366-0.86 from the Swiss National Science Foundation to M.S., by a postdoctoral fellowship from the National Institutes of Health (GM10203) to M.A.S. and a grant (GM35012) to H.F.Lodish.

References

Ashwell,G. and Harford,J. (1982) *Annu. Rev. Biochem.*, **51**, 531–554.
Baenziger,J.U. and Maynard,Y. 61980) *J. Biol. Chem.*, **255**, 4607–4613.
Bischoff,J. and Lodish,H.F. (1987) *J. Biol. Chem.*, **262**, 11825–11832.

Bischoff,J., Libresco,S., Shia,M.A. and Lodish,H.F. (1988) *J. Cell Biol.*, **106**, 1067–1074.
Bolton,A.E. and Hunter,W.M. (1973) *Biochem. J.*, **133**, 529–539.
Breitfeld,P.P., Simmons,C.F., Strous,G.J.A.M., Geuze,H.J. and Schwartz,A.L. (1985) *Int. Rev. Cytol.*, **97**, 47–95.
Bretscher,M.S. and Lutter,R. (1988) *EMBO J.*, **7**, 4087–4092.
Davis,C.G., Lehrman,M.A., Russell,D.W., Anderson,R.G.W., Brown,M.S. and Goldstein,J.L. (1986) *Cell*, **45**, 15–24.
Davis,C.G., van Driel,I.R., Russell,D.W., Brown,M.S. and Goldstein,J.L. (1987) *J. Biol. Chem.*, **262**, 4075–4082.
Drickamer,K., Mamon,J.F., Binns,G. and Leung,J.O. (1984) *J. Biol. Chem.*, **259**, 770–778.
Goldstein,J.L., Brown,M.S., Anderson,R.G.W., Russelll,D.W. and Schenider,W.J. (1985) *Annu. Rev. Cell Biol.*, **1**, 1–39.
Halberg,D.F., Wager,R.E., Farrell,D.C., Hildreth,J., Quesenberry,M.S., Loeb,J.A., Holland,E.C. and Drickamer,K. (1987) *J. Biol. Chem.*, **262**, 9828–9838.
Kornfeld,S. (1987) *FASEB J.*, **1**, 462–468.
Lazarovits,J. and Roth,M. (1988) *Cell*, **53**, 743–752.
Loeb,J.A. and Drickamer,K. (1987) *J. Biol. Chem.*, **262**, 3022–3029.
McPhaul,M. and Berg,P. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 8863–8867.
Pearse,B.M.F. (1987) *EMBO J.*, **6**, 2507–2512.
Pearse,B.M.F. (1988) *EMBO J.*, **7**, 3331–3336.
Roth,J., Bendayan,M. and Orci,L. (1978) *J. Histochem. Cytochem.*, **26**, 1074–1081.
Sawyer,J.T., Sanford,J.P. and Doyle,D. (1988) *J. Biol. Chem.*, **263**, 10534–10538.
Schneider,C., Sutherland,R., Newman,R. and Greaves,M. (1981) *J. Biol. Chem.*, **256**, 8516–8522.
Schwartz,A.L. and Rup,D. (1983) *J Biol. Chem.*, **258**, 11249–11255.
Schwartz,A.L., Bolognesi,A. and Fridovich,S.E. (1984) *J. Cell Biol.*, **98**, 732–738.
Shia,M.A. and Lodish,H.F. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 1158–1162.
Spiess,M. and Lodish,H.F. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 6465–6469.
Thompson,J.A., Lau,A.L. and Cunningham,D.D. (1987) *Biochemistry*, **26**, 743–750.
Towbin,H., Staehelin,T. and Gordon,J. (1979) *Proc. Natl. Acad. Sci. USA*, **76**, 4350–4354.
Wall,D.A. and Hubbard,A. (1981) *J. Cell Biol.*, **90**, 687–796.
Wessles,H.P., Geffen,I. and Spiess,M. (1989) *J. Biol. Chem.*, **264**, 17–20.

Received on January 9, 1989; revised on June 29, 1989

Note added in proof

After submission of this manuscript Braiterman *et al.* [Braiterman,T.L., Chance,S.C., Porter,W.R., Lee,W.C., Townsen,R.R. and Hubbard,A. L. (1989) *J. Biol. Chem.* **264**, 1682–1688] have reported a qualitative study showing that rat hepatoma tissue culture cells expressing only the major subunit of the rat ASGP receptor can internalize a synthetic ligand (*N*-acetyl-galactosylated poly-L-lysine). This finding is in agreement with our results on the human receptor.