Antibody-induced Receptor Loss
DIFFERENT FATES FOR ASIALOGLYCOPROTEINS AND THE ASIALOGLYCOPROTEIN RECEPTOR IN HEPG2 CELLS*

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The human asialoglycoprotein receptor (ASGP-R) is a membrane glycoprotein which participates in receptor-mediated endocytosis and delivery of its ligands to lysosomes for degradation. In order to examine the pathways and mechanisms responsible for the turnover and degradation of the ASGP-R we have followed the fate of the ASGP-R in HepG2 cells during exposure to anti-receptor antibody as well as inhibitors of lysosomal processing and receptor recycling. Incubation of cells at 37 °C with anti-ASGP-R antibody results in the rapid (t1/2, 30 min) loss of mature 46,000-Da ASGP-R (control, t1/2, 20 h). This process requires whole IgG, since Fab fragments do not induce loss of receptor. Furthermore, this antibody-induced loss is specific, since incubation with antibody to the transferrin receptor does not alter cellular ASGP-R content. Of note, weak bases (e.g., primaquine) abrogate this antibody-induced loss of ASGP-R. Inhibitors of lysosomal proteases (EC64 and leupeptin) do not alter this antibody-mediated loss. Furthermore, this effect occurs at 18 °C, a temperature at which delivery of ligand to the lysosome is blocked. Thus, the present observations suggest a unique pathway for antibody-induced ASGP-R loss which is distinct from the pathway of lysosomal delivery of ligand.

Plasma glycoproteins whose carbohydrate moieties terminate in galactose (asialoglycoproteins) are rapidly and efficiently cleared from the circulation by the hepatic asialoglycoprotein receptor (ASGP-R') via receptor-mediated endocytosis (for review see Ref. 1). This process is initiated by the binding of ligand to ASGP-R at the cell surface, internalization via coated pits/vesicles, and delivery to an endocytotic sorting compartment (compartment of uncoupling receptor and ligand) from which ligand is delivered to the lysosome for degradation. The ASGP-R, however, efficiently recycles back to the cell surface to undergo multiple rounds of ligand uptake (2). In addition to this major pathway of lysosomal degradation, the internalized ligand and ASGP-R also traverse at least one other distinct pathway which results in the recycling of ligand and receptor (3).

The human hepatoma cell line HepG2 provides a valuable system in which to dissect critical elements in the biosynthesis, processing, receptor-mediated endocytosis, and turnover of the ASGP-R (4). The human ASGP-R is synthesized as a 40,000-Da precursor which is converted to the 46,000-Da mature species within the Golgi upon the trimming and remodeling of the N-linked oligosaccharides (5). The mature 46,000-Da species then rapidly reaches the cell surface where it functions in ligand binding and endocytosis.

A single ASGP-R molecule may bind ligand, internalize into the cell, dissociate from its ligand within the acidic environment of the sorting compartment, and recycle back to the cell surface within 8 min (6). Dissociation of ligand from ASGP-R appears to be one crucial event which spares the ASGP-R the protease-rich lysosomal environment. Under physiological conditions in HepG2 cells the ASGP-R survives with a mean lifetime of approximately 30 h (4). Thus, under these conditions, the ASGP-R may recycle >250 times, similar to the efficiency of many other receptors which participate in receptor-mediated endocytosis (7, 8).

The intracellular site and biochemical mechanisms which lead to the degradation of the ASGP-R or other cell surface receptors are unknown. Recent studies with the Fc receptor and mannose 6-phosphate receptor suggest that these proteins are degraded in lysosomes together with their ligands (9, 10). On the other hand, Krupp and Lane (11) provided evidence that insulin and the insulin receptor traverse different pathways for degradation. However, the details of these processes are unclear.

As an experimental approach to the problem receptor degradation, we have examined the fate of the ASGP-R following exposure to anti-ASGP-R antibodies. We demonstrate herein that exposure of HepG2 cells to intact anti-ASGP-R antibody induces a rapid cellular loss of ASGP-R. This antibody-induced loss of ASGP-R is blocked by the presence of weak base, primaquine. However, the thiol protease inhibitors, leupeptin and EC64, failed to inhibit antibody-induced ASGP-R loss, despite marked inhibition of lysosomal degradation of ligand. In addition, incubation of cells at 18 °C which completely inhibits lysosomal delivery and thus degradation of ligand failed to inhibit antibody-induced ASGP-R loss. Thus, the pathway and/or mechanism(s) responsible for antibody...
induced ASGP-R loss are distinct from the lysosomal degradation of ligand.

EXPERIMENTAL PROCEDURES

Materials

Orosomucoid (a1-acid glycoprotein) was a gift from the American Red Cross laboratory. Preparation of asialoorosomucoid (ASOR) and 125I-ASOR has been previously described (12). Primaqine and saponin were from Sigma and leupeptin and EC64 were from Boehringer-Mannheim. [35S]Methionine (specific radioactivity, 1200 mCi/mmol) and 13C molecular weight standards were obtained from Amersham Radiochemicals. Immobilized papain was from Pierce Chemical Co.; Protein A-Sepharose was from Pharmacia.

Antibodies

Normal rabbit IgG was obtained from Dako. Rabbit anti-human ASGP-R antisera has been described previously (4). Human placental transferrin receptor was purified to homogeneity from fresh term human placentae according to the procedure of Seligmann et al. (13) with some modifications. Rabbit polyclonal anti-human transferrin receptor antibodies were prepared from the human placental transferrin receptor by immunizing rabbits with purified homogeneity, electroelution from SDS-PAGE, and injection into rabbits in Freund's adjuvant, similar to the procedure described for the anti-ASGP-R antibody. IgG fractions of rabbit antisera were prepared using Bio-Rad DEAE Affi-Gei blue chromatography and were assayed by SDS-PAGE under nonreducing and reducing conditions. Fab fragments were prepared from purified IgG by digestion with immobilized papain (Pierce Chemical Co.) in the presence of 20 mM cysteine. Digestion was complete as assessed by SDS-PAGE, and the Fab fragments were purified from Fc fragments by affinity chromatography over protein A-Sepharose. Evaluation of the isolated Fab fragments was performed by gel exclusion chromatography, SDS-PAGE under reducing and nonreducing conditions, and Western blotting.

Methods

Cells—The human hepatoma cell line HepG2 (clone a16) was used for all experiments. Maintenance of these cells was described in detail earlier (4).

Binding of 125I-ASOR to Cell Surface—As described previously, binding assays were performed under saturating conditions at 4°C (14). Identical aliquots of cells were processed for nonspecific binding (i.e. in the presence of 200 μg/ml nonradioactive ASOR, in the presence of 50 nM N-acetylgalactosamine, or in "stripped" cells (in which cells were stripped by incubation with PBS containing no added calcium, 10 mM EDTA at pH 5 following binding)). Specific binding is defined as the amount of 125I-ASOR bound minus the nonspecific.

Uptake and Degradation of 125I-ASOR—As described previously (14), uptake and degradation assays were carried out at temperatures greater than 4°C (see "Results"), generally at 37°C. At the appropriate times aliquots of the media were examined for the presence of 125I-ASOR degradation products as previously described (5, 14). At the end of the appropriate incubation the radioactivity which remained cell associated was determined as described previously.

Cell Permeabilization with Saponin—To determine total cellular ligand binding sites, cells were chilled by immersion in PBS at 4°C and thereafter exposed to binding media containing 1 mg/ml saponin for 30 min at 4°C (3). Following rinsing with PBS containing no calcium and with added EDTA, pH 5 (to remove endogenous ligand from receptor) the cells were incubated with 125I-ASOR under standard saturation binding conditions.

Bioassynthetic Labeling—Cells were rinsed in PBS and incubated for 15 min at 37°C with Eagle's minimal essential medium without methionine. Thereafter, [35S]methionine was added at 100–300 μCi/ml. After the appropriate time (pulse), nonradioactive methionine was added at 200–1000 X concentration of the radiolabeled methionine, and the incubation was continued for the appropriate time (chase) (4).

Immunoprecipitation of ASGP-R and TFR and Analysis by SDS-PAGE—As described previously (4), following biosynthetic labeling, the cells were immediately chilled to 4°C. The cells were then washed twice with PBS and thereafter solubilized in 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride in PBS, and the solution clarified by centrifugation (5 min, 15,000 g). To samples at 4°C, 1 volume of 1% Triton X-100, 0.5% deoxycholate, 0.5% SDS, 1 mM phenylmethylsulfonyl fluoride in PBS with 5 mg/ml human serum albumin and 5 mg/ml bovine serum albumin was added, and the samples were immunoprecipitated with the appropriate antibodies as described previously. Immunocomplexes were isolated with staphylococcal protein A, and the antigen and antibody were separated by SDS-PAGE, fixed, and prepared for fluorography with Autoradifluor.

Quantitation of Fluorograms and Western Blots—These were performed with a Helena Quanta Scan densitometer.

RESULTS

Effect of Anti-ASGP-R Antibodies on ASGP-R—In order to examine the effect of anti-human ASGP-R antibody we first determined that this antibody recognized the entire complement of ASGP-R in HepG2. We have previously demonstrated the ability of rabbit anti-human ASGP-R antibody to inhibit 125I-ASOR binding to the HepG2 cell surface (4). Since a substantial fraction of total cell ASGP-R is not accessible at the cell surface, we determined the ability of anti-ASGP-R to inhibit ligand binding to total cell ASGP-R following saponin permeabilization. As seen in Fig. 1, anti-ASGP-R inhibits 125I-ASOR binding, with 50% inhibition observed at approximately 40 μg/ml and 90% inhibition at approximately 200 μg/ml.

Next, in order to define the parameters and pathways associated with receptor turnover, we used anti-ASGP-R antibody as a probe. Preliminary experiments demonstrated that exposure of HepG2 to preimmune serum did not alter the normal processing or turnover of biosynthetically labeled ASGP-R. In contrast, addition of anti-ASGP-R to HepG2 cells at 37°C causes the loss of biosynthetically labeled 46,000-Da (mature) ASGP-R by 4 h of chase (data not shown).

![Fig. 1. Effect of anti-ASGP-R IgG on 125I-ASOR binding to permeabilized hepatoma cells.](image-url)
The kinetics of this process were examined as seen in Fig. 2. In this study, HepG2 cells were pulsed with [35S]methionine for 2.5 h and thereafter chased in the presence of unlabeled methionine for 1 h. Cells were then incubated with either preimmune or anti-ASGP-R antiserum during a further 0–120 min of chase. At the appropriate times, the cells were harvested and immunoprecipitated with anti-ASGP-R and analyzed by SDS-PAGE. As seen in the upper panel of Fig. 2, there is a time-dependent decrease in the 40,000-Da precursor species (by 70% at 120 min) and concomitant increase (by 100% at 120 min) in the 46,000-Da mature ASGP-R species. In cells exposed to anti-ASGP-R antiserum, there is a linear loss of the 40,000-Da precursor species similar to that seen in control cells. However, in contrast to the situation in control cells, there is a marked, rapid, concentration-dependent loss of the 46,000-Da mature ASGP-R species. 50% of the 46,000 Da species is lost at approximately 30–40 min. Of note, the 40,000-Da precursor appears to be converted to the 46,000 Da product prior to its loss from the cells, thus overestimating the time required for antibody-mediated ASGP-R loss. This antibody-mediated loss of ASGP-R from HepG2 cells was not a result of shedding of ASGP-R with or without membrane attachment, solubilized, immunoprecipitated with anti-ASGP-R antibody, and analyzed by SDS-PAGE under reducing conditions, boiled in 4% SDS and separated on SDS-PAGE prior to Western blotting. Alternatively, the entire contents of the culture dishes were directly boiled in SDS sample buffer prior to SDS-PAGE and Western blotting. As seen in Fig. 3, incubation with anti-ASGP-R induced a virtually complete loss of ASGP-R from the Triton X-100 lysate with a concomitant increase in ASGP-R content of the pellet. However, total ASGP-R (supernatant and pellet) decreased to approximately 10% of control. Analysis of the entire cell extract (i.e. solubilization in boiling SDS, only without centrifugation) yielded similar results (Fig. 4). In addition, under either conditions, no ASGP-R was detected at the top of gels. Thus, the antibody-induced ASGP-R loss is not a result of sequestration within a Triton-X-100-insoluble pool.

To determine whether intact IgG was responsible for the observed ASGP-R loss, both purified IgG and Fab fragments biosynthetic labeling. To reassess this apparent antibody-induced loss of ASGP-R in a manner independent of immunoprecipitation procedures, unlabeled HepG2 cells proteins were separated by SDS-PAGE under reducing conditions, transferred to nitrocellulose, and probed with anti-ASGP-R. As seen in Fig. 3 (lane a), both the 40,000-Da precursor as well as the more abundant 46,000-Da mature ASGP-R is readily visualized. Incubation of HepG2 cells with anti-ASGP-R antiserum causes a marked loss in whole cell 46,000 Da ASGP-R with no major change in the whole cell 40,000 Da precursor (Fig. 3, lane b), consistent with the observations in cells biosynthetically labeled and analyzed by immunoprecipitation.

The above experiments were performed with cells following

Fig. 2. Kinetics of anti-ASGP-R-induced loss of ASGP-R in hepatoma cells. Cells were washed with PBS and biosynthetically labeled with [35S]methionine during a 2½-h pulse period followed by a 1-h chase period as described in the text. Thereafter, either control rabbit serum (30% v/v, top left) or anti-ASGP-R serum (10%, top middle; 30%, top right) was added and the chase continued for an additional 0–120 min. At the appropriate times, the cells were harvested, solubilized, immunoprecipitated with anti-ASGP-R antibody, and analyzed by SDS-PAGE and fluorography (upper panels, with molecular mass markers noted in kDa on the left). Quantitation of these data are presented in the lower panels. The left two panels denote 10% antibody; the right two panels denote 30% antibody. The upper and lower panels represent the 46- and 40-kDa species, respectively. The symbols denote preimmune (O) or anti-ASGP-R antibody (●).

Fig. 3. Effect of anti-ASGP-R antiserum, IgG, and Fab on ASGP-R in hepatoma cells by Western blot analysis. Cells were washed in PBS and incubated with either normal rabbit serum (10%, lane a), IgG (8 µg/ml, lane c), Fab (36 µg/ml, lane e) or anti-ASGP-R serum (10%, lane b), IgG (8 µg/ml, lane d), Fab (36 µg/ml, lane f) for 4 h at 37 °C. Thereafter, whole cells were harvested and processed for Western blot analysis as described in the text. Upper panel, Western blot with molecular mass markers in kDa on the left. Lower panel, quantitation of the 46-kDa ASGP-R.
were prepared from normal rabbit serum and anti-ASGP-R antiserum (see "Methods"). As Fab fragments often possess a lower affinity than whole IgG molecules for the same antigen, we determined the bioactivity of the preparation by quantification of total activity is 80% of the sample fraction applied to each lane. For samples 1 and 2, supernatant is open bars, pellet is closed bars. For samples 3 and 4, total activity is hatched bars.

**Fig. 4. Effect of anti-ASGP-R antiserum on ASGP-R in hepatoma cells and cell extracts.** In panel A, cells were washed in PBS and incubated with either normal rabbit serum (10%) (lanes 1 and 3) or anti-ASGP-R serum (10%) (lanes 2 and 4) for 4 h at 37 °C. Thereafter, samples 1 and 2 were harvested in Triton X-100 buffer as described in the text. The supernatant (IS, 2S) and the entire pellet (IP, 2P) after boiling in 4% SDS were prepared in sample buffer. The entire contents of the culture dish for samples 3 and 4 were directly boiled in SDS-sample buffer. All samples were processed and buffer. The entire contents of the culture dish for samples 3 and 4 were directly boiled in SDS-sample buffer. All samples were processed and corrected for the sample fraction applied to each lane. For samples 1 and 2, supernatant is open bars, pellet is closed bars. For samples 3 and 4, total activity is hatched bars.

**Fig. 5. Effect of anti-ASGP-R IgG or Fab on binding of 125I-ASOR to hepatoma cells.** Cells were washed in PBS, cooled, and incubated at 4 °C in binding media with various concentrations of purified normal rabbit IgG or Fab (O) or anti-ASGP-R IgG or Fab (×). After 2 h at 4 °C, 125I-ASOR was added and saturation binding determined in the standard manner. Nonspecific binding was <15% of total binding. Each symbol represents the mean ± S.E. of determinations.

**Fig. 6. Effect of anti-ASGP-R serum, IgG and Fab on ASGP-R and Tf-R in hepatoma cells.** Cells were washed with PBS and biosynthetically labeled with [35]Smethionine during a 3-h pulse followed by a 1-h chase period. Cells were incubated with either control rabbit serum (10%, lane a), IgG (20 µg/ml, lane c), or Fab (40 µg/ml, lane e) or anti-ASGP-R serum (10%, lane b), IgG (20 µg/ml, lane d), or Fab (40 µg/ml, lane f) for 2 h at 37 °C. Thereafter, cells were solubilized and immunoprecipitated with either anti-ASGP-R (panel A) or anti-Tf-R (panel B). Molecular mass markers are in kDa on the left.
Effect of anti-ASGP-R and anti-Tf-R on the ASGP-R in hepatoma cells. Cells were washed in PBS and biosynthetically labeled with \([35S]\)methionine during a 3-h pulse followed by a 1-h chase period. Cells were then incubated with either control rabbit serum (lane a), anti-ASGP-R (lane b), or anti-Tf-R (lane c) (10%) for 4 h at 37 °C. Thereafter, cells were solubilized and immunoprecipitated with anti-ASGP-R. Molecular mass markers in kDa are on the left.

Effect of primaquine on anti-ASGP-R-induced loss of ASGP-R in hepatoma cells. Cells were washed in PBS and biosynthetically labeled with \([35S]\)methionine during a 3-h pulse followed by a 1-h chase period. Following washing, cells were then incubated in the absence or presence of 100 µM (lanes c and d) and 300 µM (lanes e and f) primaquine for 30 min at 37 °C. Thereafter, either control rabbit serum or anti-ASGP-R (15%) was added for 1 h at 37 °C. Thereafter, cells were solubilized and immunoprecipitated with anti-ASGP-R. Molecular mass markers in kDa are on the left.

Effect of Leupeptin and EC64 on Uptake and Degradation of \(^{125}\)I-ASOR in Hepatoma Cells. Cells were washed in PBS and preincubated in binding media containing various concentrations of leupeptin (○) or EC64 (●) for 1 h at 37 °C. Therefore, \(^{125}\)I-ASOR was added and the incubation continued for 6 h. Uptake and degradation were determined in the standard manner. Each value represents the mean ± S.E. of 2–3 determinations.
Antibody-induced Receptor Loss

The availability and expression of cellular receptors plays a critical role in the ability of eukaryotic cells to respond to their environment. Regulation of functional receptor molecules at the cell surface is maintained by dynamic processes which reflect the rates of synthesis, processing, distribution within the cell, and degradation. Recently, some of the details of each of these processes have been defined. For example, the pathways, kinetics, and regulation of the synthesis of many membrane proteins have been recently described (e.g., low density lipoprotein receptor, transferrin receptor, insulin receptor, asialoglycoprotein receptor (7, 8)). Abnormalities in the normal processing pathways for the low density lipoprotein receptor (18), asialoglycoprotein receptor (5), and acetylcholine receptor (19) have revealed structural features essential for physiologic function. Many recent studies on the cellular distribution of cell surface receptors have shed considerable light on the intracellular trafficking and sorting of these molecules. However, limited data are available on the pathways and mechanisms responsible for the degradation of cell surface receptors (20).

Of the receptors involved in receptor-mediated endocytosis, many appear to recycle efficiently and survive for 30-80 h (8). On the other hand, some such as the epidermal growth factor receptor appear to recycle very few if any times (21), while the IgA receptor is used once only (22). The physiological fate of the epidermal growth factor receptor may be lysosomal degradation, as is the fate of its ligand, although this has not been demonstrated conclusively (21). There are three general processes (mechanisms) which govern protein degradation: shedding from the cell, lysosomal and nonlysosomal degradation (20).

The present results provide evidence that the loss of ASGP-R following exposure to antibody is via a mechanism/pathway distinct from the classical lysosomal route. Two series of observations support this suggestion. First, the loss of ASGP-R induced by anti-ASGP-R is not inhibited by leupeptin or EC64 agents which completely inhibit lysosomal degradation of ligand. Second, the loss of ASGP-R induced by anti-ASGP-R occurs at 18°C, a temperature at which ligand is not delivered to lysosomes. These observations have been confirmed by immunoprecipitation as well as by whole cell Western blot analysis.

Other systems examined have provided additional data. For example, Mellman and Plutner (9) have demonstrated that exposure of macrophages to anti-Fc receptor caused a loss in Fc receptor. This effect was dose and time dependent and was most easily explained by misrouting of Fc receptor to the lysosome upon exposure to polyclonal antibody. Exposure to Fab fragments was without significant effect (9). Similar studies of the 215-kDa mannose 6-phosphate receptor revealed a loss of mannose 6-phosphate receptor (t 1/2, 0.5 h, compared to 10 h in control cells) (10). Fab fragments were incapable of inducing this rapid loss although a decrease in

Antibody addition 46,000-Da ASGP-R

| Antibody addition | 46,000-Da ASGP-R |
|-------------------|-----------------|
| None              | 100 ± 12        |
| Control serum     | 92 ± 12         |
| Anti-ASGP-R serum | 25 ± 2          |
| Control IgG       | 93 ± 8          |
| Anti-ASGP-R IgG   | 38 ± 4          |

60% decrease (to 38 ± 4% control) (Table II). These effects are strikingly similar to those observed at 37°C albeit during shorter incubations, i.e., in seven independent experiments anti-ASGP-R serum incubation for a mean of 2 h induced an 83% decrease (to 17 ± 6% control) and anti-ASGP-R IgG induced a 59% decrease (to 41 ± 1% control). Incubation of HepG2 cells with anti-ASGP-R for 6 h at 15°C yielded similar results (antiserum decreased to 25 ± 3% control and anti-ASGP-R IgG decreased to 46 ± 8% control (data not shown).

FIG. 11. Effect of leupeptin and EC64 on anti-ASGP-R-induced loss of ASGP-R in hepatoma cells by Western analysis. Cells were washed in PBS and incubated for 1 h at 37°C with leupeptin or EC64 at 500 µg/ml. In panel A, cells were then harvested and analyzed by Western analysis. Molecular mass markers in kDa are on the left. In panel B, control rabbit serum or anti-ASGP-R was added (20%) for 1½ h to cells with inhibitors prior to harvest and Western analysis. Quantification of the 46-kDa species is shown.

Table I

Effect of temperature on 125I-ASOR uptake and degradation in HepG2 cells at 6 h

| Temperature | Cell-associated | Degraded | Total uptake and degraded |
|-------------|----------------|----------|--------------------------|
| °C          | ng/dish (%)    |          |                          |
| 37          | 44 ± 1 (100)   | 56 ± 3 (100) | 100 ± 3 (100)       |
| 18          | 23 ± 2 (52)    | 0.3 ± 0.5 (<2) | 23 ± 2 (23)        |
| 15          | 13 ± 1 (30)    | 0.7 ± 0.2 (<2) | 14 ± 1 (14)        |

Table II

Effect of anti-ASGP-R antibody on HepG2 ASGP-R following incubation at 18°C

Cells were biosynthetically labeled with [35S]methionine with a 1-h pulse and 2-h chase in standard medium containing 25 mM Hepes (pH 7.3). After cooling cells to 18°C, antiserum (final concentration, 10%) or IgG (final concentration, 12 µg/ml) was added and the incubation continued for 6 h at 18°C. Thereafter, the cells were harvested and processed via immunoprecipitation in the standard manner. Quantification of the 46,000-Da mature ASGP-R species was performed as described under "Methods." Each value represents mean ± S.E. of 3-4 determinations.

Table III

Effect of antibody on ASGP-R uptake and degradation in HepG2 cells at 6 h

| Antibody addition | 46,000-Da ASGP-R |
|-------------------|-----------------|
| None              | 100 ± 12        |
| Control serum     | 92 ± 12         |
| Anti-ASGP-R serum | 25 ± 2          |
| Control IgG       | 93 ± 8          |
| Anti-ASGP-R IgG   | 38 ± 4          |
receptor half-life was found upon prolonged exposure (23). However, in these experiments the apparent antibody-mediated loss of the mannose 6-phosphate receptor resulted from cross-linking and subsequent detergent insolubility (23). This appears not to be the case in the present experiments, as Western blot analysis of whole cells demonstrates antibody-mediated ASGP-R loss (Fig. 4). Other studies which examined the low density lipoprotein (24) and epidermal growth factor (25) receptors following exposure of cells to intact polyclonal antibodies to the respective receptors have suggested lysosomal catabolism as the mechanism of receptor loss. The present data on the ASGP-R in hepatoma cells suggest that the classical lysosomal route is not the major degradative route under the present experimental conditions, as the proteasine inhibitors leupeptin and EC64 are ineffective as inhibitors of antibody-induced ASGP-R loss. Thus, either ASGP-R degradation is independent of the proteasines inhibited by these agents (e.g. cathepsins B and L, etc.) and/or there may be distinct populations of lysosomes, some for degradation of ligand; others for ASGP-R or nonlysosomal mechanisms are operative. The present data cannot differentiate between these possibilities. Furthermore, the observation that antibody-induced ASGP-R loss occurs at 18 °C argues against a classical lysosomal route, as there is a complete block in lysosomal delivery and degradation of ligand at this temperature (17). Recently, Mueller and Hubbard (31) have demonstrated that in hepatocytes at 16 °C ligand is internalized and remains together with ASGP-R at a prelysosomal stage.

That nonlysosomal mechanisms may be important for cell surface receptor degradation was noted by Creek and Sly (32) in studies of the mannose 6-phosphate receptor. They demonstrated that incubation with leupeptin, or NH4Cl did not alter receptor turnover. Furthermore, receptor turnover was unsalted in I-cell disease fibroblasts in which delivery to the lysosomes is defective. In addition, following insulin receptor which had been labeled with “heavy” amino acids, Krupp and Lane (33) provide evidence that neither lysosomotropic amine nor leupeptin alter insulin receptor degradation in chick liver cells.

The mechanism responsible for this phenomenon is not clear at present. Studies with the weak bases (e.g. primaquine) suggest that the ASGP-R loss occurs from a compartment distal to the earliest acidified compartment, as primaquine abrogates the antibody-mediated effect. It should be noted, however, that this apparent protection of receptor degradation by the weak bases is not likely a result of lysosomal neutralization as was thought earlier (26). More likely, the effect of these agents is a result of intracellular prelysosomal receptor sequestration (14). Thus, the ASGP-R is not exposed at the cell surface and not accessible to antibody. That is, the internalization and intracellular sequestration of cell surface proteins occurs in the presence of the weak bases irrespective of the presence of anti-ASGP-R. Thus, various interpretations may result from studies which employ the weak bases solely to neutralize lysosomal acidification.

Additional insight into the underlying mechanism of antibody-induced ASGP-R loss is suggested by the kinetics of ASGP-R loss (t1/2, 30 min). This is more rapid than the time required for ligand to reach the lysosome (6, 27). Furthermore, it is unlikely that this process results from transport along the ligand-recycling pathway, since the kinetics of ASGP-R loss are too rapid (t1/2, 30 min versus t1/2, 90 min for ligand recycling). In addition, the pathway of ligand recycling is not sensitive to primaquine (3). Thus, taken together these data are consistent with a nonlysosomal mechanism, although there is no direct evidence for this at present.

The inability of Fab fragments to induce ASGP-R loss suggests that cross-linking of ASGP-R molecules may be an important determinant in this process. The unique effects of divalent antibody may be due to antibody-induced clustering of receptors on the plasma membrane or in an intracellular sorting compartment. Since the valency of the natural ligands for the ASGP-R may vary, it is difficult to interpret how clustering may affect segregation during normal physiological cycling. Recently, van Deurs et al. (28) have demonstrated that alterations in ligand-receptor valency (i.e. of ricin conjugates) may significantly alter their intracellular pathways.

In addition, the reduced affinity of the ASGP ligand for the receptor at low pH suggests that ligand-induced clustering in acidified endocytic compartments is unlikely, since acidification of newly endocytosed vesicles appears to be a rapid event. In this regard, the differences between the binding affinities for the receptor of the ligand and the antibody, both at neutral and reduced pH, may account for the differences in observed half-lives. Thus, it will be of interest to examine antibody-induced degradation with a population of antibodies selected for low affinity at reduced pH. Effects due to other differences between Fab and IgG cannot be excluded, as for example the much greater size of intact IgG, which could restrict the movement of attached receptor in a confined intracellular space. Studies on antibody binding to receptors in other systems have suggested that bound divalent antibody directs cycling receptors to premature degradation, presumably in the lysosomes. Our evidence suggests that another pathway may exist in this system.

Thus, the present data are consistent with a mechanism or pathway for anti-ASGP-R-induced loss of ASGP-R from hepatoma cells which is distinct from the pathway of lysosomal delivery of ligand. The intracellular site and mechanism responsible for this process remain to be elucidated, as does the relationship of this process to that of other receptor polypeptides.

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