A Novel Cycle Involving Fatty Acyl-Coenzyme A Regulates Asialoglycoprotein Receptor Activity in Permeable Hepatocytes

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Asialoglycoprotein receptors (ASGP-Rs) in permeable rat hepatocytes can be inactivated in the absence of ligand. This cytosol-independent effect is relatively slow ($t_{1/2} \sim 12$ min) and is temperature and ATP dependent. Here we show that in the absence of cytosol, the addition of palmitoyl-CoA (Pal-CoA) rapidly ($t_{1/2} < 0.4$ min) and quantitatively reactivates the inactivated receptors. Receptor reactivation was half-maximal at ~ 10 – $12 \mu\text{M}$ free Pal-CoA at 37°C . Although substantially higher total concentrations were used, much of the added Pal-CoA was cell associated and not free. The effects of Pal-CoA were eliminated by bovine serum albumin at concentrations sufficient to bind all free monomeric fatty acyl-CoA, suggesting that micellar effects are not responsible for the ability to reactivate ASGP-Rs. Also, palmitoyl-carnitine did not substitute for Pal-CoA. The initial ASGP-R inactivation is not affected by treating cells with *N*-ethylmaleimide or by a KCl wash but is inhibited by sodium orthovanadate or high Ca^{2+} levels. Myristoyl-CoA (C14) was also able to reactivate inactive ASGP-Rs about as well as Pal-CoA. Fatty acyl-CoAs with chain lengths of C12 (lauroyl) or C18 (steroyl) were $<50\%$ as active. The ligand binding activity of these receptors can subsequently be modulated within minutes by the further addition of ATP or Pal-CoA to achieve additional rounds of ASGP-R inactivation or reactivation, respectively. These *in vitro* data demonstrate the occurrence of a novel asialoglycoprotein receptor inactivation–reactivation cycle that could regulate receptor activity during endocytosis and receptor recycling.

INTRODUCTION

Receptor-mediated endocytosis is a basic cellular function found in virtually all eukaryotic cells. Although there are multiple cellular pathways by which ligands bound to membrane-bound receptors are internalized, most uptake occurs via a coated pit pathway (Brown *et al.*, 1983). This pathway is employed by many growth factor and peptide hormone receptors as well as “housekeeping” receptors that receive nutrients for cells or that remove potentially harmful extracellular molecules (Drickamer, 1987). A feature common to most of these receptor systems, particularly the latter class II receptors (Anderson and Kaplan, 1983), is the recycling and reutilization of a receptor to mediate sequentially

the binding and endocytosis of hundreds to thousands of ligand molecules during its lifetime. These recycling receptors clear their ligands from extracellular fluids. Receptors such as the low-density lipoprotein, asialoglycoprotein, hyaluronan, and mannose receptors must mediate their ligand uptake and degradation efficiently, particularly when the extracellular ligand concentration is high (Weigel, 1992). Although receptor recycling has been studied extensively, it is not known how intracellular receptor routing through endosomes and back to the cell surface is achieved or regulated or why segregation of free ligand from recycling receptors is so efficient.

Asialoglycoprotein receptors (ASGP-Rs¹) in isolated rat hepatocytes mediate the endocytosis and intracel-

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¹ Abbreviations used: ASGP-R, asialoglycoprotein receptor; ASOR, asialo-orosomucoid; Pal-CoA, palmitoyl-CoA; BSA, bovine serum albumin; CMC, critical micelle concentration.

lular processing of the ligand asialo-orosomucoid (ASOR) by two functionally distinct pathways (Kindberg *et al.*, 1990; Weigel, 1992). Although 50% of the total cellular ASGP-Rs are in each pathway, the state 2 ASGP-Rs, which comprise one specific receptor population, are responsible for ~80% of the cell's ligand processing capability. Earlier studies in intact cells indicated that the state 2 ASGP-Rs were inactivated and then reactivated during receptor recycling (McAbee and Weigel, 1987). The presence of an inactivation-activation cycle regulating ASGP-R activity was supported by the observation that ASGP-R inactivation occurred in digitonin-permeabilized cells in the absence of cytosol (Medh and Weigel, 1991). These experiments demonstrated that the loss of ASGP-R ligand binding ability required ATP, did not occur at 4°C, and involved only the state 2 ASGP-R population (which represents 50% of the total cellular ASGP-R activity). This loss of receptor activity was not due to receptor degradation, to inaccessibility of receptors to ligand in the permeable cells, or to receptor occupancy (Medh and Weigel, 1991). Here we describe conditions under which the inactive state 2 ASGP-Rs can be reactivated and cycled through multiple rounds of inactivation and reactivation. This *in vitro* regulation of ASGP-R activity supports the existence of a receptor cycle involving transient inactivation and then reactivation. This new pathway has numerous implications for the function of the ASGP-R, as well as other receptors, in normal and disease states.

MATERIALS AND METHODS

Human orosomucoid, Percoll, ATP, palmitoyl carnitine, and fatty acyl-CoAs were from Sigma Chemical (St. Louis, MO). ASOR was prepared from orosomucoid and iodinated as previously described (Weigel and Oka, 1982). Na ¹²⁵I (10–20 mCi/μg of iodine) and [¹⁴C]palmitoyl-CoA (Pal-CoA) were from Amersham (Arlington Heights, IL). Digitonin was from Eastman Kodak (Rochester, NY). Bovine serum albumin (BSA) was from Intergen (Purchase, NY; grade CRG-7). Collagenase (type D) was from Boehringer Mannheim (Indianapolis, IN) and 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid was from Research Organics (Cleveland, OH). All other chemicals were reagent grade. All media and buffers have been described previously (Weigel and Oka, 1982; Oka and Weigel, 1987; Medh and Weigel, 1991).

Preparation and Permeabilization of Hepatocytes

Male Sprague-Dawley rats (200–300 g) were from Harlan Breeding Laboratories (Houston, TX). Rat hepatocytes were isolated by a collagenase perfusion procedure (Oka and Weigel, 1987). Cells were suspended in medium 1/BSA (2–3 × 10⁶ cells/ml) and incubated in a gyratory water bath at 37°C for 1 h to increase and stabilize the cell surface ASGP-R number (Weigel and Oka, 1983). Viable cells (>95%) were then selected by centrifugation (350 × g) over discontinuous Percoll gradients (Clarke and Weigel, 1985). These cells were counted, washed, and resuspended in cytosolic buffer (2 × 10⁶ cells/ml). Mild permeabilization with digitonin (Weigel *et al.*, 1983) was accomplished by resuspending in the presence of 0.006% (wt/vol) digitonin and incubating the cells at 4°C for 20 min. The permeabilized cells were centrifuged (350 × g, 2 min) and washed twice more with

cytosolic buffer to remove cytosol and then resuspended in cytosolic buffer to 2 × 10⁶ cells/ml unless noted otherwise.

ASGP-R Activity Assays

Permeable cells were incubated during experiments at either 4 or 37°C with continuous gyration at 100 rpm (usually, 2.5 ml in a 25 ml Erlenmeyer flask). Samples of 1 ml were diluted at different times into 5 ml of ice-cold cytosolic buffer. The cells were then centrifuged, and the supernatant removed by aspiration. Cell pellets were resuspended and incubated at 4°C for 1 h in 0.5 ml of medium 1/BSA with 1.5 μg/ml ¹²⁵I-ASOR (specific activity, ~50–100 cpm/fmol) and 0.055% digitonin. The presence of 0.055% digitonin fully permeabilizes the cells and allows one to measure total cellular (surface and internal) receptor number and activity (Weigel *et al.*, 1983). The cells were washed twice with 8 ml Hanks', the final cell pellets were resuspended in 0.5 N NaOH, and aliquots were assessed for radioactivity. Non-specific ¹²⁵I-ASOR binding, determined by the bound radioactivity remaining in the presence of a 75-fold excess of nonlabeled ASOR or after stripping specifically bound ligand with ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid, was only 5–10% of the total in all cases (Weigel and Oka, 1982). All binding assays were done in duplicate, and the values differed on average by ~5%.

ASGP-R Inactivation

Permeable washed hepatocytes were incubated in cytosolic buffer containing 5–7.5 mM ATP at 37°C for 60 min (Medh and Weigel, 1991). The cells were chilled by dilution into three volumes of cytosolic buffer on ice and centrifuged. The cell pellet was resuspended in cytosolic buffer to 2 × 10⁶/ml. Cell samples were removed before and after the ATP treatment at 37°C and ligand binding activity was determined as above at 4°C. Under these conditions, 40–60% of the total cellular ¹²⁵I-ASOR binding activity is lost (Medh and Weigel, 1991). This value in each experiment is defined as 100% of the ASGP-R inactivation.

General

Cell viability was determined by Trypan blue exclusion. Fatty acyl-CoAs were dissolved in dimethyl sulfoxide as 15 mM stock solutions. Final dimethyl sulfoxide concentrations were usually ≤1% (vol/vol). Controls for solvent addition alone showed no effect on ASGP-R activity. Protein content was measured by the method of Bradford (1976) using BSA as a standard. Radioactivity was determined using a Packard (Meriden, CT) Multiprias 2γ spectrometer (for ¹²⁵I) or a Beckman (Fullerton, CA) LS7500 liquid scintillation spectrometer (for ¹⁴C) and Bio-Safe II scintillation fluid (from Research Products).

RESULTS

We previously found (Medh and Weigel, 1991) that when permeable washed cells are incubated with 5–7.5 mM ATP at 37°C, state 2 ASGP-R activity is lost in ~30–60 min (*t*_{1/2} ~ 12 min). Because the relatively slow rate of the initial ASGP-R inactivation is consistent with a requirement for receptor or membrane translocation or even vesicle fusion, we examined the effect of several treatments known to affect these processes. For example, we employed agents known to remove or inactivate several of the peripheral membrane proteins involved in endosomal vesicle fusion (Malhotra *et al.*, 1988; Clary *et al.*, 1990). Washing the permeable cells with KCl or treating them with the sulfhydryl reagent *N*-ethylmaleimide did not decrease their ability to inactivate ASGP-Rs (Table 1). However, the initial slow

Table 1. Effect of various treatments on the initial ASGP-R inactivation by ATP in permeabilized rat hepatocytes

Treatment/condition	ASGP-R inactivation (% of control)
None (ATP alone)	100
2 mM <i>N</i> -ethylmaleimide	85
5 mM <i>N</i> -ethylmaleimide	95
0.5 M KCl wash	98
20°C	0
3 mM sodium vanadate	41
5 mM sodium vanadate	27
1 mM calcium chloride	0
5 mM γ -methylene-ATP (no ATP)	0

Cells were permeabilized, washed, and incubated at 37°C with 5 mM ATP (unless noted otherwise) plus the indicated agent. After 45 min, the cells were chilled to 4°C, washed twice in cytosol buffer, and assayed for ASGP-R activity as described in MATERIALS AND METHODS. The ASGP-R activity before incubation was used as the maximum activity (i.e., 0% inactivation) and the number of ASGP-Rs inactivated with 5 mM ATP alone was set equal to 100%. Results are averages of duplicate determinations.

ASGP-R inactivation did not occur at $\leq 20^\circ\text{C}$ or in the presence of vanadate or high levels of Ca^{2+} . These results indicate that protein kinases or Ca^{2+} -regulated proteins may be involved in the initial inactivation process. ASGP-R inactivation caused by ATP was not affected by 40 μM triascin C, which can inhibit Pal-CoA synthesis (Pfanner *et al.*, 1989). This result indicates that fatty acyl-CoAs are not involved in the receptor inactivation process. Ongoing experiments also indicate that one of the three receptor subunits, RHL1, is transiently phosphorylated during this initial inactivation (Haynes *et al.*, 1992). The ATP requirement could be for receptor translocation or inactivation or both.

After washing away the ATP, the complete cohort of inactivated ASGP-Rs could be quickly reactivated by the addition of Pal-CoA (Figure 1). The restoration of ligand binding activity was dependent on Pal-CoA concentration and appeared to require relatively high concentrations. Unlike the initial ATP-dependent inactivation step, which takes >30 min (Medh and Weigel, 1991), ASGP-R reactivation was surprisingly fast. At 200 μM Pal-CoA, virtually all the inactive ASGP-Rs ($\sim 2 \times 10^5/\text{cell}$) were reactivated within 30 s. Even at lower concentrations of Pal-CoA (25–100 μM) maximal reactivation occurred within 1 min at 30°C . Other experiments have shown that free palmitate and Co-A will not substitute for Pal-CoA (Weigel and Oka, 1993).

The apparent concentration of Pal-CoA needed for ASGP-R reactivation in Figure 1 is relatively high. However, because the above experiments use ~ 1 mg of membrane protein/assay (~ 0.5 mg protein/ 10^6 permeable cells), this large amount of protein could absorb most of the added Pal-CoA and greatly decrease the actual free concentration. The capacity of membranes,

or BSA, to bind fatty acyl-CoAs is well known (Brecher, 1983; Perez-Gil *et al.*, 1990; Das *et al.*, 1992). We used [^{14}C]Pal-CoA to assess this possibility by determining the partition of fatty acyl-CoA between the cells and medium (Figure 2A). Using 2×10^6 cells/ml and varying the Pal-CoA concentration, 79% of the added [^{14}C]Pal-CoA was cell associated after 2 min at 37°C . Separate kinetic experiments showed that the maximum amount was associated with the cells by 2 min. Furthermore, as expected the fraction of Pal-CoA that partitions into the cells is dependent on the cell concentration (Figure 2A). At 1.0×10^6 cells/ml, 49% of the [^{14}C]Pal-CoA was cell associated. Therefore, the permeable cells can sequester most of the exogenous Pal-CoA. Thus, with 2×10^6 cells/ml and 150 μM Pal-CoA as in Figure 1, the actual free concentration in the medium (i.e., monomeric and micellar Pal-CoA) is only ~ 30 μM . Another potential concern with the use of apparently high concentrations of Pal-CoA is the possible detergent-like effects of Pal-CoA. However, very little solubilization of cellular membrane protein occurred with increasing concentrations of Pal-CoA either at 1 or 2×10^6 cells/ml, suggesting that micelles are not present (Figure 2B). Furthermore, palmitoyl-carnitine, which has similar detergent properties to Pal-CoA but is not an acyl donor in palmitoylation reactions, was not as effective in reactivating ASGP-Rs (Figure 3). At 75 μM , Pal-CoA was greater than fivefold more effective than palmitoyl-car-

Figure 1. Pal-CoA reactivates asialoglycoprotein receptors previously inactivated in vitro with ATP. Hepatocytes were permeabilized for 20 min at 4°C with 0.006% digitonin in cytosolic buffer, washed twice to remove cytosol, suspended in cytosol buffer (2×10^6 ml), and treated with 7.5 mM ATP for 75 min at 37°C to inactivate ASGP-Rs (Medh and Weigel, 1991). The cells were washed with cytosol buffer at 4°C and then incubated at 30°C with 0 (○), 10 (●), 25 (▽), 50 (▼), 100 (□), or 200 (■) μM Pal-CoA. ASGP-R activity was determined on washed cell samples by measuring ^{125}I -ASOR binding as described in MATERIALS AND METHODS. Specific binding determined in the presence of excess unlabeled ASOR was typically $\sim 90\%$. The Y-axis represents all (100%) of the ASGP-Rs inactivated by the first ATP treatment. This was about half of the initial total cellular ASGP-R activity.

Figure 2. Effect of Pal-CoA concentration on protein retention and free Pal-CoA. Permeable washed cells were incubated at 1.0×10^6 (Δ) or 2.0×10^6 ($\bullet$) cells/ml with the indicated concentrations of [^{14}C]Pal-CoA (final specific activity of 305 cpm/nmol). After 2 min at 37°C the cells were centrifuged (undiluted, 2 min, $80 \times g$) and radioactivity (A) and protein content (B) in the supernatant medium and cell pellet were then determined. The slopes in A, determined by linear regression analysis ($\text{ccs} > 0.99$), were used to calculate that 49% (Δ) and 79% ($\bullet$) of the added [^{14}C]Pal-CoA was cell associated.

nitine. Above about 80–100 μM , palmitoyl-carnitine appeared to solubilize more cellular protein than Pal-CoA.

Because the cells can sequester most of the Pal-CoA, the effect of a given concentration of Pal-CoA should increase as the cell concentration decreases, because the effective free Pal-CoA concentration should increase. This is the observed result (Figure 4). The ability of a given concentration of Pal-CoA to reactivate ASGP-Rs was greater when the cell concentration was decreased from 2 to 1×10^6 cells/ml. The apparent K_m for ASGP-R reactivation based on total Pal-CoA concentration were 15 and 70 μM , respectively, using 1.0 and 2.0×10^6 cells/ml. Therefore, the need for a high Pal-CoA concentration is due to the relatively high cell number used. Based on the results in Figures 2A and 4, the dose response for ASGP-R reactivation was determined using

Figure 3. Effect of palmitoyl-carnitine on ASGP-R reactivation. Permeable washed cells were prepared and ASGP-Rs inactivated as in Figure 1. These cells were washed and incubated either with 50 or 75 μM Pal-CoA for 2 min ($\square$) or 4 min ($\blacksquare$) or with 50 or 75 μM palmitoyl-carnitine (Carn) for 2 min ($\blacksquare$) or 4 min ($\square$). The samples were then chilled, washed, and ASGP-R activity was determined. Two separate experiments, assessed at 2 or 4 min and performed with different cell preparations, are shown.

the measured free Pal-CoA concentration (Figure 5). The different dose responses at different cell concentrations (seen in Figure 4) became identical when normalized in this way with an apparent K_m for ASGP-R reactivation of 10.6 μM free Pal-CoA at 37°C . This is similar to the reported K_m values for vesicle fusion and budding systems (Pfanner *et al.*, 1989, 1990; Comerford and Dawson, 1993) and for many fatty acyl transferases (Ito *et al.*, 1987; Bakken and Farstad, 1992; Coleman *et al.*, 1992).

Figure 4. Effect of cell number on ASGP-R reactivation with Pal-CoA. Permeable washed cells were prepared and ASGP-Rs inactivated as in Figure 1. These cells were washed, suspended to 1.0 (Δ) or 2.0 ($\bullet$) $\times 10^6$ cells/ml, and incubated with the indicated concentrations of Pal-CoA at 37°C . After 2 min the cells were washed and ASGP-R activity was determined. The values are plotted vs. total Pal-CoA concentration and are normalized to the maximum reactivation in each experiment. The curves were calculated by second-order regression analysis.

Figure 5. Effect of free Pal-CoA concentration on ASGP-R reactivation. The same ASGP-R activity data in Figure 3 are plotted vs. free Pal-CoA. The free Pal-CoA concentrations were calculated based on the slope of the curves for cell-associated Pal-CoA at 1.0 (▲) and 2.0 (●) $\times 10^6$ cells/ml as shown in Figure 2A.

Because the critical micelle concentration (CMC) for Pal-CoA is 40–80 μM (Powell *et al.*, 1981, 1985; Constantinides and Steim, 1985; Smith and Powell, 1986; Das and Hajra, 1992), most of the free Pal-CoA should be monomeric rather than in micelles in the experiments reported here. Consistent with this above interpretation, we find that the inclusion of ≤ 10 μM BSA (0.067%) does not inhibit ASGP-R reactivation with 150 μM Pal-CoA (Figure 6A). Because BSA binds at least one equivalent of Pal-CoA (Perez-Gil *et al.*, 1990), it competes with the membranes for sequestering the fatty acyl-CoA. As the BSA concentration increases, the amount of [^{14}C]-Pal-CoA that is cell associated also decreases (Figure 6B). The cell-associated fraction of Pal-CoA (at 150 μM) was decreased by $\sim 50\%$ in the presence of 16 μM BSA.

A variety of fatty acyl-CoA derivatives of different chain lengths were also tested for the ability to support the reactivation of ASGP-Rs (Table 2). The butyl-(C4) and decanoyl-(C10) CoAs were inactive. Pal-CoA (C16) and myristoyl-CoA (C14) gave roughly equal ASGP-R reactivation after 3 min. Lauroyl-CoA (C12) and stearyl-CoA (C18) showed comparable ASGP-R reactivation but only to about half the level seen with Pal-CoA. The kinetics of ASGP-R reactivation with the active fatty acyl-CoAs were similar to that with Pal-CoA, although some differences were observed (Weigel and Oka, 1993). Therefore, the ability of a fatty acyl-CoA derivative to mediate ASGP-R reactivation is dependent on the fatty acyl chain length.

A noticeable feature of the kinetics of ASGP-R reactivation, particularly at lower Pal-CoA concentrations, is the biphasic nature of the response (Figure 1). ASGP-R activity increases, plateaus for a variable time, and then decreases at rates inversely proportional to the Pal-

CoA concentration. One interpretation of this result is that ASGP receptors continually go through an inactivation–reactivation cycle in which receptor activation is mediated by the exogenously added Pal-CoA and receptor inactivation is mediated by residual ATP. (ATP levels, measured by a luciferase assay, in such washed cells are not reduced below ~ 10 μM .) Under these conditions of high Pal-CoA and low ATP concentrations, the steady-state operation of such an ASGP-R inactivation–reactivation cycle appears to favor the formation of active receptors. If this interpretation is correct, then subsequent addition of either ATP or more Pal-CoA should shift this distribution, respectively, to less or more active receptors. This was exactly the observed result (Figure 7). After an initial inactivation of ASGP-Rs in the presence of ATP, the cells were washed and treated with 150 μM Pal-CoA, which reactivated $\sim 70\%$

Figure 6. Effect of BSA on ASGP-R reactivation with Pal-CoA. (A). ASGP-R reactivation in permeable cells (2×10^6 /ml) was determined after 4 min at 37°C using 150 μM Pal-CoA as in Figure 1 in the presence of increasing concentrations of BSA (which was previously dialyzed vs. cytosolic buffer). (B) Permeable cells (2×10^6 /ml) were incubated with 150 μM [^{14}C]Pal-CoA as in Figure 2 and various concentrations of BSA. After 2 min at 37°C, cell suspensions were centrifuged and cell-associated radioactivity was determined.

Table 2. Effect of fatty acyl chain length on ASGP-R reactivation

Fatty acyl-CoA (chain length)	ASGP-R reactivation (%)
None	0
Butyl (4)	0
Decanoyl (10)	1
Lauroyl (12)	46
Myristoyl (14)	81
Palmitoyl (16)	78
Stearoyl (18)	37

Hepatocytes were permeabilized and treated with ATP as in Figure 1 to inactivate ASGP-Rs. The washed cells were then incubated at 37°C with 150 μ M fatty acyl-CoA derivatives of different chain lengths as indicated. After 3 min the cells were chilled and ASGP-R activity was determined as in Figure 1.

of these receptors within 2 min. When ATP was then added either 3 or 10 min after the addition of Pal-CoA, all of the ASGP-R activity that had been reactivated by the addition of Pal-CoA was again lost within 1–2 min. Conversely, if the incubation with Pal-CoA was allowed to continue for 20 min (by which time the fraction of reactivated receptors had decreased by 50%) and then a second addition of Pal-CoA was made, there was a sharp increase in ASGP-R reactivation (Figure 7).

DISCUSSION

Our results show, for the first time, a possible role for Pal-CoA in regulating the ligand binding activity of a recycling membrane receptor. The results provide evidence for a novel regulatory cycle by which the activity of the ASGP-R is turned off and on. The observed kinetics are consistent with an initial slow ATP-driven inactivation process that brings all of the cellular state 2 ASGP-Rs to a compartment or point at which receptor inactivation can occur. That this initial ASGP-R inactivation reflects a physiological relevant process and requires ASGP-R or membrane translocation is indicated by its sensitivity to vanadate and Ca^{2+} and a requirement for temperatures above 20°C.

The subsequent ASGP-R reactivation is ~ 40 times faster than the first inactivation ($t_{1/2} = 15\text{--}30$ s vs. 12 min), suggesting that any factors acting on the ASGP-R and the receptors themselves are now co-localized in the permeable cells. Only the addition of fatty acyl-CoA is needed; translocation of ASGP-Rs or other components no longer seems required. Likewise, subsequent rounds of ASGP-R inactivation driven by ATP also occur almost immediately. A large number of cellular ASGP-Rs are involved in this activity cycle ($\sim 2 \times 10^5$ /cell; $\sim 50\%$ of the total ASGP-R pool), and they are initially distributed throughout the recycling endocytic pathway in the live cell (Hubbard *et al.*, 1979; Weigel, 1992). The rapid cycling between active and inactive forms of the receptor strongly suggests that, after the

first inactivation process, all the necessary factors (e.g., enzymes) for both processes are membrane bound and in the same subcellular compartment. No soluble cytosolic factors are needed in vitro for this inactivation-reativation cycle.

An important concern in this study has been to understand why an unusually high Pal-CoA concentration was required for the observed effects on ASGP-R activity. Our initial conditions (using 2×10^6 cells per assay point) allowed us to obtain good duplicates and to measure $\leq 10\%$ changes in ligand binding activity with confidence. However, hepatocytes are very large cells (1 mg protein/ 10^6 intact cells) with a high membrane content. Our results clearly show that the permeable cells sequester a large majority of the added Pal-CoA. The actual free Pal-CoA concentrations that affect ASGP-R activity are in a range (< 25 μ M) that indicates the occurrence of specific biochemical, rather than detergent, effects. Consistent with this interpretation, palmitoyl-carnitine was not as effective as Pal-CoA in reactivating ASGP-Rs.

The free Pal-CoA concentrations in these experiments are below the reported CMC value of 40–80 μ M (Powell *et al.*, 1981, 1985; Constantinides and Steim, 1985; Smith and Powell, 1986; Das and Hajra, 1992). The results with BSA also indicate that the CMC has not been reached. The number of Pal-CoA molecules per micelle is between 40 and 200 (Powell *et al.*, 1981). Therefore, 3.75 μ M BSA could bind the equivalent of ≥ 150 μ M Pal-CoA, if the lipid were present predominantly in micelles, but only 3.75 μ M if the Pal-CoA is monomeric (BSA is reported to bind one equivalent of Pal-CoA) (Perez-Gil *et al.*, 1990). The lack of inhibition by ≤ 10 μ M BSA (Figure 6A) indicates that most of the Pal-CoA is monomeric rather than micellar. With 30

Figure 7. Asialoglycoprotein receptors can cycle between active and inactive forms. Permeable washed hepatocytes were treated with ATP as in Figure 1 to inactivate ASGP-Rs. These permeable cells were then washed and incubated with 150 μ M Pal-CoA (●). After 3 or 10 min, 7.5 mM ATP (○) was added (open arrows) to one set of samples. At 20 min, a second addition of 150 μ M Pal-CoA (■) was made to another set of samples. The filled arrows indicate the two Pal-CoA additions. ASGP-R activity was determined as in Figure 1.

μM BSA, however, ASGP-R reactivation was $\sim 50\%$ inhibited at 4 min. Complete inhibition of ASGP-R reactivation occurred with $74 \mu\text{M}$ BSA. Because at the higher BSA concentrations, essentially all the free Pal-CoA could be monomeric and still be sequestered by the BSA, elimination of the receptor reactivation reaction is expected. We conclude from these above results that the ability of Pal-CoA to reactivate ASGP-Rs in permeable hepatocytes is not due to micelle or detergent-like effects. The apparently high concentration of Pal-CoA required is directly due to the large cell mass employed.

Although its mechanism of action is unknown, Pal-CoA is required for some vesicle fusion and vesicle budding processes (Pfanner *et al.*, 1989, 1990; Comerford and Dawson, 1993) and can modulate Ca^{2+} levels in permeable cells (Deeney *et al.*, 1992). Numerous membrane proteins and receptors also have been shown to be palmitoylated, often at Cys residues near transmembrane domains, although functional consequences of these posttranslational modifications have been difficult to determine (Sefton and Buss, 1987; Olson, 1988; Grand, 1989). The subunits of the rat and human ASGP-Rs have a Cys-Ser sequence close to the cytoplasm membrane junction (Spiess *et al.*, 1985; Halberg *et al.*, 1987). This same sequence in the transferrin receptor is palmitoylated at Cys (Omary and Trowbridge, 1981; Adam *et al.*, 1988). It is possible, therefore, that the same position in one or more of the ASGP-R subunits is also palmitoylated. In a preliminary experiment, affinity-purified ASGP-Rs were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis after treatment of cells for 3 min with $50 \mu\text{M}$ [^{14}C]Pal-CoA. Under the conditions reported here, only one of the three receptor subunits, RHL1, became radiolabeled. These experiments are difficult, however, because the required autoradiographic exposure time was 6 mo. Further studies on the molecular basis for the Pal-CoA effect on ASGP-R activity are in progress.

The molecular basis for the ATP-dependent ASGP-R inactivation process is also presently unknown. Although stable phosphorylation of receptor does not occur, we have observed transient phosphorylation of Tyr5 in RHL1 in permeable washed cells incubated with ^{32}P -ATP (Haynes *et al.*, 1992). The other two ASGP-R subunits, RHL2 and RHL3, are not phosphorylated. Because phosphorylation of RHL1 is transient, it is difficult to determine whether this event is secondary or required for subsequent ASGP-R inactivation. However, inhibition of RHL1 phosphorylation with tyrphostin also blocks ASGP-R inactivation, suggesting that the two processes are causally linked.

The discovery of a new pathway by which the activity of endocytic ASGP-Rs may be regulated is significant for several reasons. First, the receptor inactivation-reativation cycle provides a clear molecular basis for the high endocytic efficiency of the ASGP-R system.

After the internalization of receptor-ligand complexes, transient receptor inactivation will allow the as yet uncharacterized segregation process to separate free ligand from receptor. The efficiency of delivering endocytosed ligand to lysosomes for degradation rather than non-productively back to the extracellular medium would be increased by preventing the rebinding of ligand to receptor during this process. Although the low pH of endosomes may contribute to this segregation by decreasing the affinity between ASGP-R and ligand (Harford *et al.*, 1983; Tycko *et al.*, 1983), there are numerous reasons why low pH alone cannot explain the observed efficiency (Schwartz *et al.*, 1985; Davoust *et al.*, 1987; Weigel, 1992). Receptor inactivation would allow the segregation process to be independent of ligand concentration. Because ligand concentrations within endosomes can be $\sim 10^4$ -fold greater than outside the cell (Weigel, 1992), this transient inactivation would allow the ASGP-R system to function well even when extracellular and intracellular ligand concentrations were high.

Second, we believe that many, and perhaps all, of the other recycling receptors may also be regulated by an inactivation-reativation cycle during their recycling itinerary. Although the molecular mechanisms by which different receptor activities are regulated could vary, the presence of a transient inactivation step would ensure that the ligands they recognize are also effectively cleared and processed. Initial experiments with human HepG2 hepatoma cells have also shown ATP-dependent ASGP-R inactivation and then subsequent reactivation in the presence of Pal-CoA. The human ASGP-R system, therefore, also appears to undergo an inactivation-reativation cycle. Most of the other endocytic recycling receptors that have been well studied, including the mannose (Kaplan and Keogh, 1981; Tietze *et al.*, 1982) and low-density lipoprotein (Basu *et al.*, 1981) receptors, show the same functional characteristics in intact cells that led us to investigate the ASGP-R inactivation-reativation cycle. Inactivation-reativation cycles may be a fundamental part of the spatial, temporal, and biochemical pathway by which recycling receptors function.

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