Lysosomal Accumulation of the Hormone-Receptor Complex during Receptor-mediated Endocytosis of Human Choriogonadotropin

MARIO ASCOLI
Division of Endocrinology, Departments of Medicine and Biochemistry, Vanderbilt University Medical School, Nashville, Tennessee 37232

ABSTRACT The experiments presented herein were designed to determine the fate of the human choriogonadotropin (hCG) receptor during endocytosis of the receptor-bound hCG. Using several biochemical approaches, it is shown that the receptor is internalized together with the hormone into endocytic vesicles and transferred to lysosomes without ligand dissociation. Once delivered to the lysosomes, the hCG-receptor complex dissociates, and the free hormone is degraded. This pathway appears to prevent receptor recycling and probably promotes receptor degradation.

Recent studies on the process of receptor-mediated endocytosis have shown that the intracellular pathway followed by the ligand–receptor complex is not always the same. In most cases, the internalized ligand–receptor complex is sorted in such a way that the ligand is transported to the lysosomes, where it is usually degraded, whereas the receptor recycles back to the cell surface while bound to the receptor (3, 4). Yet, a third example involves those complexes where the ligand is degraded, and the receptors do not appear to recycle (5–8). In this last category, the involvement of lysosomes in ligand degradation is well documented (5–11), and the receptor also appears to follow the same fate (8, 12, 13).

Human choriogonadotropin (hCG) is a glycoprotein hormone that has been shown to be degraded by target cells. We have previously characterized the fate of this ligand in Leydig tumor cells and have shown that, although hCG is internalized and degraded by a pathway whose biochemical properties closely resemble those of receptor-mediated endocytosis (6, 10, 11), the hCG receptors do not appear to recycle back to the surface (7).

The experiments reported herein were designed to investigate the fate of the receptor during the endocytosis of receptor-bound hCG. By using several biochemical approaches, it is shown that the receptor is internalized together with hCG, and that after internalization the hormone-receptor complex is transferred to endocytic vesicles to lysosomes without ligand dissociation. Once the complex reaches the lysosomes, it dissociates, and the hormone is degraded. Although it is not known if the receptor is also degraded, the finding that it reaches the lysosomes, where it can potentially undergo degradation, may provide an explanation for the apparent lack of receptor recycling observed in this system (7).

MATERIALS AND METHODS

Supplies: Purified hCG (Batch #CR-123) was obtained from the National Institute of Child Health and Human Development and labeled with $^{125}$I as described elsewhere (10). All tissue culture supplies were obtained from Gibco Laboratories (Grand Island, NY). Tissue culture plastic ware was purchased from Costar (Cambridge, MA) or Falcon Plastics, Div. of Becton, Dickinson Co. (Oxnard, CA). Crude hCG (3,000 U/mg), leupeptin, phenylmethylsulfonyl fluoride, bovine serum albumin (Cohn fraction V), Trasylol, polyethylene glycol 8,000, Triton X-100, monensin, bovine γ-globulins, nitroblue tetrazolium, and ferric chloride were purchased from Sigma Chemical Co., (St. Louis, MO). HEPES, 3-[(3-cholamidopropyl)dimethyl-ammonio]-1-propanesulfonate (CHAPS), BSA, and human transferrin were from Calbiochem-Behring Corp. (San Diego, CA). Percoll was from Pharmacia (Uppsala, Sweden), and all electrophoresis supplies from Bio-Rad Laboratories (Richmond, CA). Na$^{125}$I was purchased from Amersham Corp. (Arlington Heights, IL).

Cell Culture: The origin and handling of the MA-10 cells have been described (14). Experimental cultures were plated on 6-cm dishes on day 0 (split ratio 1:7) and used on day 3 when the number of cells/dish was ~4 x 10^6.

1 Abbreviations used in this paper: CHAPS, 3-[(3-cholamidopropyl)dimethyl-ammonio]-1-propanesulfonate; hCG, human choriogonadotropin.
All experiments were done in Waymouth MB752/1 modified to contain 20 mM HEPES and 1 mg/ml albumin pH 7.4 (assay medium). The assay medium used for the 37°C incubations also contained 6.75 mM NaHCO₃, and the incubations were done in a humidified atmosphere of 95% air/5% CO₂. For the 4°C incubations, the NaHCO₃ was replaced with an equimolar concentration of NaCl, and CO₂ was provided by a humidifier.

All incubations were initiated by placing the cells on ice for 30 min. After washing them twice with cold assay medium (2 ml/wash) each dish received 2 ml of cold assay medium and 20 ng/ml ¹²⁵I-hCG alone, or together with 25 IU/ml of hCG (to correct for nonspecific binding). After a 2-h incubation at 4°C, the cells were washed five times (2 ml/wash) with cold Hanks' balanced salt solution containing 1 mg/ml albumin (wash medium), and processed immediately (t = 0) or incubated at 37°C in assay medium for the time indicated in the figures.

Analysis of Internalization, Degradation, and Dissociation of the Internalized Hormone-Receptor Complex: At the times indicated in the figures, the dishes were placed on ice, and the medium was saved and precipitated with 10% trichloroacetic acid (6). The soluble radioactivity was taken as a measurement of degraded hormone. The cells were washed once with cold wash medium and incubated for 2-4 min with cold 50 mM glycine/100 mM NaCl, pH 3. The radioactivity removed by this wash was taken as a measurement of the surface-bound hormone, and the radioactivity that remained associated with the cells was collected after solubilization with 0.5 N NaOH and considered to be internalized hormone (10).

The assay used to determine the proportion of intracellular radioactivity that remains receptor-bound is based on the ability of polyethylene glycol to precipitate this complex without precipitating the free hormone (15). After the acid wash (see above), the cells were washed twice with 2-ml portions of cold assay medium, scraped into a minimal amount of the same medium, and recovered by centrifugation (the contents of two dishes were combined). Each pellet was then resuspended in 150 µl of 1% (wt/vol) Triton X-100, 0.15 M NaCl, 20 mM HEPES, pH 7.4, supplemented with 20 mM leupeptin, 1 mM phenylmethylsulfonyl fluoride, and 100 µg/ml Trasylol and incubated for 45 min at 4°C. The extracts were diluted 10-fold with the same buffer without Triton X-100 and centrifuged at 100,000 g for 1 h. The supernatants were then collected and aliquots were counted. Other aliquots were precipitated with polyethylene glycol, and the precipitates were collected by centrifugation and counted. It was previously shown that, when cells containing only surface-bound ¹²⁵I-hCG are treated in this manner, ~85% of the cell-associated radioactivity is precipitated and solubilized in 1% Triton X-100. This precipitate is defined as 100% of receptor-bound activity and used to correct all the internalized samples.

Analysis of Internalized Radioactivity on Percoll Gradients: After the acid treatment (see above), the cells were washed twice with cold assay medium and twice with homogenization medium (0.25 M sucrose, 10 mM HEPES, 1 mM EDTA, pH 7). The cells were scraped from the dishes into the homogenization medium, and the contents of four dishes were combined, centrifuged by resuspension in 2 ml of homogenization medium and homogenized with a motor-driven Teflon pestle at 5,000 rpm (five strokes). Postnuclear supernatants were prepared by centrifuging the homogenates at 800 g for 10 min. The supernatants were saved and the pellets resuspended and centrifuged again. The two supernatants were combined, and a 2-ml aliquot was thoroughly mixed with 8 ml of a solution of Percoll (in homogenization medium) with a density of 1.065 g/ml (16) and centrifuged at 10,000 g for 60 min (70 Ti rotor) in a Beckman L3-50 ultracentrifuge (Beckman Instruments, Inc., Fullerton, CA). The contents of the gradients were collected from the top with the aid of a Buchler autodensiflow. In some experiments, the contents of the first five fractions were combined, mixed with 1 ml of homogenization medium, and fractionated on a second gradient. 2-ml aliquots of the pooled fractions were then mixed and rehomogenized (8 ml of Percoll density = 1.044 gm/ml) and centrifuged at 33,000 g for 20 min (16). The gradients were fractionated as described above.

Further Analysis of Gradient Fractions: After running and fractionating the first gradient, the fractions containing endocytotic vesicles and plasma membranes (fractions 1-5) and those containing lysosomes (fractions 29-35) were not combined. No attempt was made to separate endocytotic vesicles from plasma membranes, because it was shown that the majority of the radioactivity present in this pool in the internalized samples is not associated with plasma membranes (cf. Fig. 6).

To determine if the internalized radioactivity associated with each of these organelles was receptor-bound, each pool received protease inhibitors (see above), and 100 µl of 10% (wt/vol) Triton X-100. The volume was adjusted to 2 ml with homogenization buffer, and the samples were incubated for 45 min at 4°C. The samples were then diluted two-fold with homogenization buffer and centrifuged at 100,000 g for 60 min to pellet the Percoll and insoluble material. The supernatants were precipitated with polyethylene glycol (see above) to determine the percentage of the solubilized radioactivity that remained receptor-bound. This procedure solubilized 70-75% and 92-97% of the radioactivity present in the plasma membranes (fractions 1-5) and those containing lysosomes (fractions 29-35), respectively. A sample containing only surface-bound ¹²¹I-hCG carried through these manipulations showed that ~70% of the radioactivity solubilized from each pool was precipitated with polyethylene glycol. This value was defined as 100% of receptor-bound activity and used to correct all the internalized samples.

In other experiments, the pooled fractions were extracted as above, except that CHAPS (final concentration = 10 mM) was used instead of Triton X-100. The supernatants were then diluted two-fold with 200 mM glycine, pH 3, and incubated for 4 min at 4°C to dissociate the receptor-bound ¹²¹I-hCG (11, 14). The samples were then neutralized with 0.5 N NaOH, dialyzed against H₂O, lyophilized, and analyzed by SDS PAGE as described elsewhere (10). Under the electrophoretic conditions used, ¹²¹I-hCG does not dissociate into subunits unless the samples are heated before electrophoresis (10). A constant amount of radioactivity (1,000–4,000 cpm) was applied to each lane. After electrophoresis, the gels were dried and the radioactive bands were visualized by autoradiography. Autoradiograms were obtained by exposing the dried gel (~70°C, 24–72 h) to preflashed Kodak XAR-5 film (Eastman Kodak Co., Rochester, NY) with Cronex Hi-Plus intensifying screens (E. I. Du Pont & Co., Wilmington, DE) (17). After autoradiography, the radioactive bands were excised and counted.

A similar protocol was used to solubilize the total internalized radioactivity associated with intact cells. After extraction, aliquots of the solubilized cells were precipitated with polyethylene glycol (see above), the pellets were redissolved in 5 mM CHAPS, and acidified and processed as above for analysis on SDS gels. This procedure permitted analysis of the total internalized radioactivity and the internalized radioactivity that remained receptor-bound.

Measurements of Receptor Occupancy and Down-Regulation: Dishes (6 cm) were preincubated on ice for 30 min and washed twice with 2-ml portions of cold assay medium. Each dish then received 2 ml of cold assay medium and increasing concentrations of hCG. After a 4-h incubation at 4°C, the free hormone was removed by washing the dishes five times with 2-ml portions of cold Hanks' balanced salt solution containing 1 mg/ml of albumin, pH 7.4. The dishes then received 2 ml of cold assay medium and a saturating concentration of ¹²¹I-hCG alone or together with 25 IU/ml crude hCG (to correct for nonspecific binding) and incubated at 4°C for 2 h. The free ¹²¹I-hCG was then removed by washing (see above), and the cells were solubilized with 0.5 N NaOH (10) and counted in a Beckman Instruments 4000 Gamma Counter. Because the rate of dissociation of the prebound hCG is negligible (10, 15), and all incubations were done at 4°C, the decrease in ¹²¹I-hCG binding observed as a function of the amount of hCG used in the 4°C preincubation is a measure of occupancy of the cell surface receptors.

Receptor down-regulation was measured under conditions where the cells can perform one or several rounds of endocytosis of hCG. Experiments involving one or more than one round of endocytosis were initiated by incubating the cells with increasing concentrations of hCG at 4°C. After the 4°C incubation, the cells were not washed; they were simply transferred to a 37°C incubator, incubated for 18 h, cooled to 4°C for 30 min, washed twice, treated with 4°C, 2 ml with isotonic glycine, pH 3 to remove residual surface-bound hormone (10), and then used to measure ¹²¹I-hCG at 4°C (see above).

Because there is no free hormone present during the 37°C incubation, the cells can internalize and degrade only the hormone that was initially bound. It was previously shown that during this time the cells internalize and/or degrade over 90% of the prebound hCG (10) and that the residual surface-bound hormone is efficiently reprocessed without affecting the activity of the receptor (10, 18, 19). Thus, the decrease in ¹²¹I-hCG binding observed as a function of the amount of hCG used in the 4°C preincubation is a measure of receptor occupancy of the cell surface receptors.

To determine receptor down-regulation under conditions where the cells can perform one or several rounds of endocytosis of hCG, experiments involving several rounds of endocytosis were also initiated as above, except that at the end of the 4°C preincubation the cells were not washed; they were simply transferred to a 37°C incubator, incubated for 18 h, cooled to 4°C for 30 min, washed twice, treated with 4°C, 2 ml with isotonic glycine, and then used to measure ¹²¹I-hCG binding at 4°C (see above).

Since, during the 37°C incubation, there is free hormone present in the medium, the cells will internalize and degrade the prebound hCG and can continue to bind, internalize, and degrade the free hCG present. Thus, the decrease in ¹²¹I-hCG binding observed as a function of the amount of hCG present throughout the experiment is a measure of receptor down-regulation during several rounds of endocytosis.

Other Methods: β-Hexosaminidase activity was measured in the presence of 0.5% Triton X-100 as described by Hall et al. (20). Adenylate cyclase...
activity was measured in the presence of 10^{-8} M forskolin as described by Smith and Limbird (21).

Diferric transferrin was prepared as described by Klausner et al. (22). This ligand was labeled by mixing 150 μg of protein with 1 μCi of ^125^INa and 50 μg of chloramine T (in a total volume of 70 μl of 0.1 M potassium phosphate buffer, pH 7.2) for 25 s at room temperature. The reaction was stopped by adding 100 μg of NaS2O4 (in 10 μl of the same buffer) and the mixture was separated (at 4°C) on a 0.9 × 27-cm column of Sephadex G-75 equilibrated and eluted with the same buffer. The specific activity obtained was ~7 × 10^{9} cpm/μg.

^125^I-Diferric transferrin was used to label the endocytic vesicles of the MA-10 cells because the intracellular route followed by this ligand is such that little, if any, of the internalized ligand is delivered to the lysosomes (3, 4, 23). Before doing these experiments, we showed that the MA-10 cells have transferrin receptors that mediate the uptake of this ligand by the pathway described in other cells (3, 4, 23). In addition, a new method was developed to remove the surface-bound transferrin. This method is based on the binding that the rate of dissociation of apotransferrin at neutral pH is faster than that of diferric transferrin (3, 4). Thus, to remove the surface-bound transferrin, the dishes were placed on ice, washed, and incubated at 4°C for 10 min in 2 ml of 25 mM sodium acetate, 150 mM NaCl, and 50 μM desferrioxamine, pH 5.0 (to remove the iron bound to transferrin) and then incubated at 4°C for 20 min in 2 ml of wash medium containing 10 μg/ml diferric transferrin and 50 μM desferrioxamine (to allow dissociation of the bound apotransferrin). The radioactivity removed during the last incubation was considered to be surface-bound, whereas the that remaining cell-associated was considered to be internalized. Control experiments showed that this treatment removed 90% of the ^125^I-diferric transferrin bound to the MA-10 cells at 4°C.

To label the endocytic vesicles, cells were incubated with ^125^I-diferric transferrin (2 μg/ml) for 2 h at 4°C. After washing, the cells were incubated at 37°C for 15 min with diferric transferrin, placed on ice, and treated as described above to remove the surface-bound ligand. Postnuclear supernatants were then prepared and analyzed as described above. Under these conditions, 50% of the initially bound ^125^I-transferrin had been internalized and returned to the medium intact, 25% was bound to the cell surface, and 25% was internalized.

RESULTS

Internalization and Degradation of the Receptor-bound hCG

A single round of endocytosis of the receptor-bound ^125^I-hCG can be monitored by preincubating the MA-10 cells with the hormone at 4°C, washing to remove the free hormone, and incubating the cells in hormone-free medium at 37°C for up to 4 h. When this is done, the cells internalize and degrade over 90% of the hormone that was initially bound (Fig. 1A). It should be noted that in comparison with other ligands the rate of internalization of the surface-bound ^125^I-hCG is relatively slow (t^1/2 = 20–30 min, see references 7 and 11), and the lag observed before the appearance of degradation products in the medium (i.e., ~60 min) is longer (1, 2).

The experiment shown in Fig. 1B was designed to determine if the internalized ^125^I-hCG remained receptor-bound. This was done by washing the cells with acid to remove the surface-bound hormone, followed by detergent extraction of the intracellular radioactivity, and precipitation of the receptor-bound radioactivity with polyethylene glycol. The data show that there was an initial increase in the internal receptor-bound ligand which reached a maximum at 30 min, and then declined. The free internal ligand increased more slowly; it reached a maximum at 2 h, and then declined. These results, together with the time course of appearance of degradation products in the medium (cf. Figure 1A), suggest a precursor-product relationship whereby the hormone–receptor complex is internalized in the intact form and dissociates intracellularly before the onset of ligand degradation.

It should be stressed that, in this and subsequent experiments, we followed the fate of the hormone-receptor complex (not the free receptor) and the free hormone by differential precipitation with polyethylene glycol. The validity of this method was previously documented by the following findings (15): (a) When cells are incubated with ^125^I-hCG at 4°C, washed to remove the free hormone, and solubilized with Triton X-100, 80–90% of the radioactivity bound is recovered in soluble form. (b) When the detergent extracts prepared as described above are treated with polyethylene glycol, at least 86% of the radioactivity is precipitated. (c) If the detergent extracts prepared as described above are acidified (to dissociate the hormone–receptor complex, see reference 10) before adding polyethylene glycol, only 4% of the radioactivity is precipitated. (d) If the detergent extracts prepared as described above are incubated at neutral pH and 4°C for 24 h, there is little or no decrease in the amount of radioactivity precipitated with polyethylene glycol.

Thus, it is clear that our experimental conditions extract the hormone–receptor complex without dissociating it, that this complex is stable at neutral pH and low temperature, and that the polyethylene glycol assay precipitates the complex without appreciable precipitation of the free hormone.

Effects of NH₄Cl, Monensin, and Leupeptin

Previous experiments from this laboratory have shown that NH₄Cl and monensin, compounds that raise the pH of endocytic vesicles and lysosomes (24, 25), as well as leupeptin,
an inhibitor of lysosomal proteases (26, 27), inhibit the degradation of the internalized hCG but do not affect binding or internalization (6, 10, 11, 28). It was therefore of interest to determine if these agents also had an effect on the dissociation of the internalized hormone-receptor complex. As shown in Fig. 2, it was found that these three compounds blocked the intracellular degradation of hCG and the dissociation of the internalized hormone-receptor complex to about the same extent. In general, however, the inhibitory effect of leupeptin on the dissociation of the ligand-receptor complex was somewhat less than the effects NH4Cl or monensin on this process.

The close correlation observed between the inhibition of ligand degradation and dissociation of the ligand–receptor complex suggests that most of the internal receptor-bound radioactivity may be undegraded hormone. This possibility was tested directly by analyzing the total and receptor-bound intracellular radioactivity on SDS polyacrylamide gels. The results presented in Fig. 3 show that the total intracellular radioactivity isolated from control cells is composed of intact hCG (apparent Mr = 42,500) and two degradation products of the hormone (apparent Mr = 34,000 and 19,000). The total intracellular radioactivity isolated from cells treated with NH4Cl, monensin, or leupeptin, however, is composed primarily of intact hCG. The data presented in Fig. 3 also show that the receptor-bound intracellular radioactivity is primarily intact hCG even in the absence of inhibitors of ligand degradation.

The effects of these inhibitors on dissociation of the intracellular hCG–receptor complex are somewhat different than those reported for the asialoglycoproteins, where NH4Cl and monensin have been shown to inhibit complex dissociation, while leupeptin was found to have no effect (29–31).

**Density-Gradient Centrifugation**

Recent studies from several laboratories have shown that the plasma membranes, endocytic vesicles, and lysosomes can be readily separated on Percoll gradients (16, 29). Thus, we utilized this experimental approach to gain additional information about the intracellular locations of dissociation of the ligand–receptor complex and ligand degradation.

The protocol used is similar to that of Merion and Sly (16), where a postnuclear supernatant is applied to a first Percoll gradient to separate endocytic vesicles and plasma membranes from lysosomes. The gradient fractions containing the endocytic vesicles and plasma membranes are then applied to a second Percoll gradient that allows the separation of these two latter compartments.

The results presented in Fig. 4 show the density profiles of the two gradients and the position of biochemical markers for plasma membranes (adenylate cyclase), endocytic vesicles (internalized transferrin), and lysosomes (β-hexosaminidase). In the first gradient (left panels), the majority of the plasma membrane and endocytic vesicles migrated to the top (ρ ≤ 1.05 g/ml), whereas most of the lysosomes banded at a density of 1.08–1.09 g/ml. When the five first fractions of the first gradient were combined and centrifuged on a second gradient, the following results were observed:

- **Control Cells:**
  - The receptor-bound intracellular radioactivity remains intact hCG.
  - The dissociation products (Mw = 42,500; 34,000; and 19,000) are present.

- **NH4Cl (10 mM), Monensin (25 μM), or Leupeptin (200 μM):**
  - The receptor-bound intracellular radioactivity remains intact hCG.
  - The dissociation products (Mw = 42,500; 34,000; and 19,000) are present.

**FIGURE 3** SDS gel electrophoresis of the internalized hCG. Cells were incubated with hCG, as described in Fig. 1, and processed after 1 h of internalization. Where indicated, NH4Cl (10 mM), monensin (25 μM), or leupeptin (200 μM) were present throughout the experiment. After removing the surface-bound hCG, the cells were extracted with CHAPS and treated with polyethylene glycol to precipitate the receptor-bound radioactivity. Aliquots of the untreated extracts (total intracellular radioactivity) or the polyethylene glycol precipitates (receptor-bound intracellular radioactivity) were then analyzed on a SDS polyacrylamide gel, as described in Materials and Methods. The numbers on the left show the position of molecular weight markers. A constant amount of radioactivity was applied to each lane. The results of a representative experiment are shown.
gradients is in agreement with previous data on the existence of two distinct populations of lysosomes in other cell types (16, 32) and also show that the experimental conditions used result in minimal lysosomal damage.

The analysis of the surface-bound and internalized [125I]-hCG on the first Percoll gradient is shown in Fig. 5. All the surface-bound [125I]-hCG (A) migrated to the top of the gradient, whereas the internalized radioactivity (B–E) was resolved into two compartments. The position of the less dense component is identical to that of the only peak of surface-bound hormone and corresponds to the position of plasma membranes and endocytic vesicles. Because the samples containing only surface-bound or internalized hormone were treated differently (see Materials and Methods, and the legend to Fig. 5), the radioactivity present in this area should be associated with different cellular structures: plasma membranes in A and endocytic vesicles in B–E (this contention is shown to be true in Fig. 6).

The more dense component shown in Fig. 5 contains most of the internalized radioactivity (B–E) and co-migrates with β-hexosaminidase. Although only one time point is presented, other experiments done at earlier time points (not presented) show that, as time increases, the internalized radioactivity moves from the less dense (i.e., endocytic vesicles) to the more dense (i.e., lysosomes) compartment. The data presented in Fig. 5 also show that leupeptin has no effect on the transfer of radioactivity from endocytic vesicles to lysosomes, whereas NH₄Cl and monensin have a weak inhibitory effect. Thus, in the presence of these two compounds, there is a 60% increase in the radioactivity associated with endocytic vesicles and a 30% decrease in the radioactivity associated with lysosomes. These results stand in contrast with those obtained with a number of other ligands (16, 29–31), where monensin and NH₄Cl have been shown to completely prevent the transfer of ligand to lysosomes.

Fig. 6 shows the results obtained when the first five fractions from the gradients shown in Fig. 5 were applied to the second Percoll gradient. These data show that the majority of the surface-bound hormone co-migrated with the plasma membrane marker, whereas the majority of the internalized radioactivity migrated as a single peak with a density of ~1.045 g/ml. Note that, although this peak coincides with the position of the peak of internalized transferrin (cf. Fig. 4), the distribution of internalized [125I]-hCG is more localized to this peak than the distribution of internalized transferrin. The results presented in Fig. 6 also show that NH₄Cl, monensin, and
Further Analysis of the Radioactivity Associated with Endocytic Vesicles and Lysosomes

Taken together, the data presented suggest that the internalized hCG-receptor complex dissociates not in the endocytic vesicles but rather in the lysosomes, where the liberated hCG is degraded. To obtain direct evidence for this hypothesis, we isolated and analyzed endocytic vesicles and lysosomes for the presence of receptor-bound and degraded ¹²⁵I-hCG.

The results presented in Fig. 7 show that, when analyzed on SDS polyacrylamide gels, most of the radioactivity associated with endocytic vesicles migrated as intact ¹²⁵I-hCG. This was true in cells incubated with or without inhibitors. It should be noted, however, that a small amount of the first degradation product was detectable in the control and NH₄Cl-treated cells. In the control cells most of the lysosomal radioactivity associated with lysosomes was degraded ¹²⁵I-hCG, whereas in the cells treated with inhibitors most of the radioactivity remained undegraded. Thus, it appears that little (if any) degradation occurs in the endocytic vesicles, and that all inhibitors tested prevent hormone degradation in the lysosomal compartment.

The intracellular location of dissociation of the hormone-receptor complex was determined using polyethylene glycol precipitation of detergent extracts of endocytic vesicles and lysosomes. The data presented in Table I clearly show that dissociation of the hormone-receptor complex occurs in the lysosomes and that NH₄Cl, monensin, and leupeptin prevent this phenomenon. Thus, it appears that the hCG-receptor complex does not dissociate in the endocytic vesicles but is delivered to the lysosomes intact. Once in the lysosomes, the complex dissociates and the free hormone is degraded. Inasmuch as we cannot yet follow the fate of the free receptor, it

leupeptin had no effect on the profiles of the internalized ¹²⁵I-hCG.

Further Analysis of the Radioactivity Associated with Endocytic Vesicles and Lysosomes

Cells were incubated with ¹²⁵I-hCG as described in Fig. 1 and processed after 1 h of internalization. Where indicated, NH₄Cl (10 mM), monensin (25 µM), or leupeptin (200 µM) were present throughout the experiment. After removing the surface-bound ¹²⁵I-hCG, the cells were homogenized and postnuclear supernatants were prepared and centrifuged on the first Percoll gradient. The fractions containing endocytic vesicles (i.e., fractions 1–5, cf. Fig. 5) and lysosomes (i.e., fractions 25–35, cf. Fig. 5) were pooled and extracted with CHAPS, as described in Materials and Methods. The extracts were then analyzed on a SDS polyacrylamide gel as described in Materials and Methods. A constant amount of radioactivity was applied to each lane. The numbers on the left show the position of molecular weight standards.

Table I

| Inhibitor    | Endocytic vesicles | Lysosomes |
|--------------|--------------------|-----------|
| None         | 97 ± 3             | 27 ± 1    |
| NH₄Cl (10 mM)| 95 ± 2             | 74 ± 5    |
| Monensin (25 µM)| 99 ± 1       | 97 ± 3    |
| Leupeptin (200 µM)| 99 ± 1       | 60 ± 4    |

Cells were incubated with ¹²⁵I-hCG as described in Fig. 1 and processed after 1 h of internalization. The indicated inhibitors were present throughout the experiment. After removing the surface-bound hormone, the cells were homogenized and postnuclear supernatants were prepared and centrifuged on the first Percoll gradient (cf. Fig. 5). The fractions containing endocytic vesicles and lysosomes were pooled, extracted with Triton X-100, and precipitated with polyethylene glycol, as described in Materials and Methods.
is not known if it remains in the lysosomes and is degraded or transported to another cellular compartment.

Because the acidic pH of the endocytic vesicles is believed to be responsible for the dissociation of other ligand–receptor complexes (2–4, 22, 23, 25, 29–31, 33, 34), it was of interest to determine if the hCG–receptor complex is stable when exposed to an acidic environment. Thus, we tested the effect of pH on the dissociation of the cell-bound 125I-hCG and compared the results with those obtained with 125I-transferrin, a ligand that does not dissociate from its receptor in endocytic vesicles (3, 4, 22, 23). The results presented in Table II show that the transferrin–receptor complex, the hCG–receptor complex does not dissociate until the pH is lowered to about 3. Thus, at least in theory, the hCG–receptor complex can survive the acidic environment (pH = 5) that prevails in endocytic vesicles.

Further Studies on the Fate of the Receptor

To gain additional information about the fate of the receptor, we examined the relationship between receptor occupancy and down-regulation. Two groups of cells were incubated at 4°C with increasing concentrations of hCG to occupy increasing amounts of the surface hCG receptors and then washed to remove the free hormone. The degree of receptor occupancy was then determined in one group of cells during a second incubation at 4°C with a fixed, saturating concentration of 125I-hCG. The other groups of cells was incubated in hormone-free medium for 4 h at 37°C to allow the occupied receptors to internalize and degrade the bound hormone. This latter group was then cooled to 4°C and treated to remove residual surface-bound hormone before determining 125I-hCG binding at 4°C. A comparison of 125I-hCG binding as a function of the amount of hCG used in the initial 4°C incubation between both groups of cells should thus provide a relationship between receptor occupancy and down-regulation after one round of endocytosis.

As shown in Fig. 8, the degree of receptor down-regulation is proportional to the degree of occupancy, but the magnitude of down-regulation is less than that predicted by occupancy. Thus, when 95% of the cell surface receptors are occupied by hCG and engaged in endocytosis, the net loss of cell surface hCG receptors is only ~50%. These results show that during a single round of endocytosis some active hCG receptors are replenished at the cell surface.

Table II

| pH   | 125I-hCG | 125I-Transferrin |
|------|---------|-----------------|
| % of initial cpm bound | % of initial cpm bound |
| 7.48 | 100     | 91              |
| 5.88 | 98      | 92              |
| 4.68 | 74      | 101             |
| 4.15 | 74      | 99              |
| 2.97 | 16      | 23              |
| 2.54 | 13      | 19              |

Cells were incubated with 125I-hCG (40 ng/ml) or 125I-differic transferrin (2 µg/ml) for 2 h at 4°C. After washing, some dishes were saved to determine the amount of radioactivity bound, and the rest were incubated for 5 min at 4°C in medium adjusted to the indicated pH. The medium was then removed, and the cells were washed once with medium of the appropriate pH before measuring the cell-bound radioactivity.

The experiment shown in Fig. 8B was designed to determine the relationship between occupancy and down-regulation during several rounds of endocytosis. The design of this experiment was similar to that described above, except that down-regulation was measured under conditions that allow several rounds of endocytosis to occur. Thus, after occupancy of the cell surface receptors, the free hormone was not removed, and the cells were incubated for 18 h at 37°C before removing the residual surface-bound hormone and testing for 125I-hCG binding. The results presented showed that under these conditions the degree of down-regulation exceeded the initial level of occupancy, presumably because in the continuous presence of free hormone the cell surface receptors continued to bind and internalize the hormone until all the receptors were down-regulated.

Taken together, these results show that during endocytosis of receptor-bound hCG there is a replenishment of some cell surface receptors and that such receptors are capable of binding and internalizing additional hormone. Extensive down-regulation occurs after several rounds of endocytosis because the replenishment of cell surface receptors is not complete, and ultimately, the rate of disappearance of receptors exceeds the rate at which receptors appear at the cell surface (7).
In addition, we tested the effects of NH₄Cl, monensin, and leupeptin on the replenishment of surface receptors shown in Fig. 8A and found that only monensin inhibited this process (data not shown). Inasmuch as all these compounds effectively inhibit the lysosomal dissociation of the internalized hormone–receptor complex and the lysosomal degradation of the internalized hormone (cf. Figs. 2, 3, and 7, and Table I), it can be concluded that these processes are not involved in receptor replenishment. These results suggest that the receptors that are replenished at the cell surface are not derived from the pool of internalized receptors (i.e., there is no receptor recycling) but are derived from an independent pool of receptors. The possibility that some receptors are recycled, however, cannot be excluded.

**DISCUSSION**

Previous results from this laboratory have shown that upon binding to its cell surface receptor, the bound hCG is internalized and degraded by a pathway whose biochemical properties closely resemble those of receptor-mediated endocytosis (6, 10, 11, 28). The experiments presented herein provide additional evidence that the process of hCG degradation occurs by this pathway. Thus, it is now shown that (a) the internalized hormone can be localized in two distinct intracellular compartments (i.e., endocytic vesicles and lysosomes) and (b) the degradation of the internalized hormone occurs in the lysosomes.

By using a combination of previously published procedures (10, 15, 16, 29), we have also been able to indirectly follow the fate of the hormone–receptor complex during the internalization and degradation of the receptor-bound hCG. The data presented are consistent with a model where the receptor is internalized together with hCG into endocytic vesicles and transferred into lysosomes without ligand dissociation. Once in the lysosomes, the complex dissociates, and the free hormone is degraded. The fate of the free receptor could not be clearly established, but, like the hormone, it also appears to be degraded rather than recycled back to the surface.

Although not formally shown, it is assumed that the internalized hCG–receptor complex encounters an acidic environment in the endocytic vesicles (23, 25, 31, 33, 35). The lack of dissociation of this complex in endocytic vesicles may be explained by the finding that, like the transferrin–receptor complex (3, 4), the hCG–receptor complex does not readily dissociate at pH 5. Unlike the transferrin–receptor complex (3, 4, 22), however, the hCG–receptor complex is delivered to the lysosomes intact rather than recycled to the cell surface.

Once in the lysosomes, the hormone–receptor complex dissociates, and the free hormone is degraded. Both of these processes can be effectively inhibited with monensin, NH₄Cl, and leupeptin. Thus, in the presence of these compounds, the hormone is internalized at a normal rate, and the hormone–receptor complex accumulates in the lysosomes, but it is not dissociated and the hormone is not degraded. The ability of leupeptin to inhibit the dissociation of the hCG–receptor complex suggests that the action of lysosomal proteases (on the hormone and/or the receptor) plays a role in this event. Inasmuch as the effect of leupeptin on the dissociation of the complex is somewhat less than the effects of NH₄Cl and monensin (cf. Fig. 2 and Table I), it is also likely that this event is aided by the acidic intralysosomal pH. It is also important to note that, in contrast to previous results obtained with other ligands (16, 29–31), we found that NH₄Cl and monensin have little effect on the transfer of the hCG–receptor complex to the lysosomes (cf. Fig. 5).

The fate of the free receptor is not known. Because it is ultimately located in the lysosomes where it can potentially be degraded (as the hormone is), it is likely that the pathway described here prevents receptor recycling, promotes receptor degradation, and is ultimately responsible for down-regulation.

I thank Byron Glenn for excellent technical assistance and Steve Johnson and Dr. Lee Limbird for performing the adenylate cyclase assays. I also thank Dr. Lee Limbird and Dr. Deborah Segaloff-Ascoli for helpful suggestions and for reading this manuscript.

This work was supported by a grant from the National Cancer Institute (CA-23603).

Received for publication 20 April 1984, and in revised form 2 July 1984.

**REFERENCES**

1. Goldstein, J. L., R. G. W. Anderson, and M. S. Brown. 1979. Coated pits, coated vesicles, and receptor-mediated endocytosis. Nature (London). 279:478-485.
2. Brown, M. S., R. G. W. Anderson, and J. L. Goldstein. 1983. Recycling receptors: the round trip itinerary of migrant membrane proteins. Cell. 32:663-667.
3. Dautry-Varsat, A., A. Ciechanover, and H. F. Lodish. 1983. pH and the recycling of transferrin during receptor-mediated endocytosis. Proc. Natl Acad. Sci. USA. 80:2258-2262.
4. Klauser, R. D., G. Ashwell, J. van Renswoude, J. B. Harford, and K. R. Bridges. 1983. Binding of apotransferrin to K562 cells: explanation of the transferrin cycle. Proc. Natl Acad. Sci. USA. 80:2263-2266.
5. Carpenter, G., and S. Cohen. 1976. 125I-labeled human epidermal growth factor: binding, internalization and degradation. J. Cell Biol. 71:159-171.
6. Ascoli, M., and D. Puett. 1978. Degradation of receptor-bound human choriongonadotropin by murine Leydig tumor cells. J. Biol. Chem. 253:4892-4899.
7. Lloyd, C. E., and M. Ascoli. 1983. On the mechanisms involved in the regulation of the cell surface receptors for human choriongonadotropin and mouse epidermal growth factor in cultured Leydig tumor cells. J. Cell Biol. 96:321-326.
8. Millman, I. S., H. Plattner, R. M. Steinman, J. C. Unkeless, and Z. A. Cohn. 1983. Internalization and degradation of macrophage Fc receptors during receptor-mediated phagocytosis. J. Cell Biol. 96:887-895.
9. Haigler, H. T., J. A. McKanna, and S. Cohen. 1979. Direct visualization of the binding and internalization of a ferritin conjugate of epidermal growth factor in human carcinoma cells A431. J. Cell Biol. 84:382-395.
10. Ascoli, M. 1982. Internalization and degradation of receptor-bound human chorion- gonadotropin in Leydig tumor cells: fate of the hormone subunits. J. Biol. Chem. 257:13306-13311.
11. Ascoli, M., and D. Puett. 1978. Inhibition of the degradation of receptor-bound human chorion- gonadotropin by monensin, probenecid, and leupeptin. J. Biol. Chem. 253:7832-7838.
12. Stoecheck, C. M., and G. Carpenter. 1984. Down regulation of Epidermal Growth Factor (EGF) receptors: direct demonstration of receptor degradation in human fibroblasts. J. Cell Biol. 98:1048-1053.
13. Kanuga, M., C. R. Kahn, J. A. Hedo, E. van Obberghen, and K. M. Yamada. 1981. Insulin-induced human lymphocytes: the relationship of receptor degradation to accelerated receptor degradation. Proc. Natl Acad. Sci. USA. 78:6967-6972.
14. Ascoli, M. 1981. Characterization of several clonal lines of cultured Leydig tumor cells: gonadotropin receptors and steroidogenic responses. Endocrinology. 103:R3-R5.
15. Ascoli, M. 1983. An improved method for the solubilization of stable gonadotropin receptors. Endocrinology. 113:2125-2134.
16. Mamon, M., and W. S. Shy. 1983. The role of intermediate vesicles in the adsorptive endocytosis and transport of ligand to lysosomes by human fibroblasts. J. Cell Biol. 96:464-466.
17. Laskey, R. A. 1980. The use of intensifying screens or organic scintillators for visualizing radioactive molecules resolved by gel electrophoresis. Methods Enzymol. 65:363-371.
18. Freeman, D. A., and Ascoli, M. 1981. Desensitization to gonadotropins in Leydig tumor cells: involvement of gonadotropin receptors and decreased capacity for steroidogenesis. Proc. Natl Acad. Sci. USA. 78:6309-6313.
19. Segaloff, D. L., and M. Ascoli. 1981. Removal of the surface-bound human chorion- gonadotropin receptors results in the cessation of hormonal responses in cultured Leydig tumor cells. J. Biol Chem. 256:14230-14233.
20. Hall, C. W., J. I. Lieberans, P. D. Lepine, and E. Neufeld. 1978. Enzymatic diagnosis of the genetic mucopolysaccharide storage diseases. Methods Enzymol. 50:439-456.
21. Smith, S. K., and L. E. Limbird. 1982. Evidence that human platelet α-adrenergic receptors coupled to inhibition of adenylate cyclase are not associated with the subunit of adenylate cyclase ADP-ribosylated by cholera toxin. J. Biol. Chem. 257:10471-10478.
22. Klauser, R. D., J. V. Renswoude, G. Ashwell, C. Kempf, A. N. Schachter, A. Dean, and K. R. Bridges. 1983. Receptor-mediated endocytosis of transferrin in K62 cells. J. Biol. Chem. 258:4715-4724.
23. van Renswoude, J., K. R. Bridges, J. B. Harford, and R. D. Klauser. 1982. Receptor-mediated endocytosis of transferrin and the uptake of Fe in K62 cells: identification of a non-lysosomal acidic compartment. Proc. Natl Acad. Sci. USA. 79:6186-6190.
24. Okuhara, S., and B. Poole. 1978. Fluorescence probe measurement of the intralysosomal pH in living cells and the perturbation of pH by various agents. Proc. Natl Acad. Sci. USA. 75:3327-3331.
25. Maschfeld, F. 1982. Weak bases and ionophores rapidly and reversibly raise the pH of endocytic vesicles in cultured mouse fibroblasts. J. Cell Biol. 93:676-681.
26. Szego, C., B. J. Seeler, and R. E. Smith. 1976. Lysosomal cathepsin B1: partial characterization in rat preputial gland and recompartmentation in response to Estradiol 17β. Eur. J. Biochem. 69:463-474.
27. Barrer, A. J., and M. F. Heath. 1977. Lysosomal Enzymes. In Lysosomes. A Laboratory Handbook. J. T. Dingle, editor. Elsevier/North-Holland, Inc., New York. 19-146.
28. Ascoli, M. 1979. Inhibition of the degradation of receptor-bound human chorionic gonadotropin by leupeptin. Biochim. Biophys. Acta 586:608–614.
29. Harford, J., K. Bridges, G. Ashwell, and R. D. Klausner. 1983. Intracellular dissociation of receptor-bound asialoglycoproteins in cultured hepatocytes. A pH-mediated nonlysosomal event. J. Biol. Chem. 258:319–3197.
30. Harford, J., A. W. Wolkooff, G. Ashwell, and R. D. Klausner. 1983. Monensin inhibits intracellular dissociation of asialoglycoproteins from their receptor. J. Cell Biol. 96:1824–1828.
31. Tycko, B., C. H. Keith, and F. R. Maxfield. 1983. Rapid acidification of endocytic vesicles containing asialoglycoprotein in cells of a human hepatoma line. J. Cell Biol. 97:1762–1776.
32. Rome, L. H., A. J. Garvin, M. M. Allieta, and E. F. Neufeld. 1979. Two species of lysosomal organelles in cultured human fibroblasts. Cell. 17:143–153.
33. Tycko, B., and F. R. Maxfield. 1982. Rapid acidification of endocytic vesicles containing α2-macroglobulin. Cell. 28:643–651.
34. Geuze, H. J., J. W. Slot, G. J. A. M. Strous, H. Lodish, and A. L. Schwartz. 1983. Intracellular site of asialoglycoprotein receptor-ligand coupling: double-label immunoelectron microscopy during receptor-mediated endocytosis. Cell. 32:277–287.
35. Merson, M., P. Schleisnger, R. M. Brooks, J. M. Moehring, T. J. Moehring, and W. S. Sy. 1983. Defective acidification of endosomes in Chinese hamster ovary cell mutants “cross-resistant” to toxins and viruses. Proc. Natl. Acad. Sci. USA. 80:5315–5319.