Internalization and Degradation of Receptor-bound Human Chorionic Gonadotropin in Leydig Tumor Cells

FATE OF THE HORMONE SUBUNITS*

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The studies presented herein were aimed at characterizing the pathway involved in the internalization and degradation of human chorionic gonadotropin by cultured Leydig tumor cells.

A quick biochemical method that differentiates between the surface-bound and internalized hormone was developed. Using this method and two hormone derivatives labeled exclusively (with 125I) in the α or β subunits, it was possible to follow the fate of each hormone subunit during hormone binding, internalization, and degradation.

The results show that the hormone is internalized in the intact form and that it reaches its place of degradation (presumably the lysosomes) in the intact form. The pathway for degradation of the internalized hormone is complex, and it appears to involve processing of one or both subunits of the intact hormone, followed by subunit dissociation and further degradation of the individual subunits. The α subunit is quickly degraded by the cells. The only detectable degradation products are extracellular amino acids. The β subunit is degraded slower, and several intracellular degradation products are detectable before amino acids appear in the medium.

It is now well established that the binding of several protein ligands to specific cell-surface receptors is followed by the internalization and/or degradation of the bound protein (reviewed in Refs. 1-4). The process of internalization has been visualized using fluorescent (4), ferritin-labeled (5, 6), gold-labeled (7), and 125I-labeled ligands (8). Results from such experiments have delineated a pathway (called receptor-mediated endocytosis) that involves the internalization of the bound ligand via coated pits and vesicles and subsequent accumulation in the lysosomes. The involvement of the lysosomes in the degradation of the internalized ligand has also been documented by biochemical methods (6, 9).

Previous studies from this (10-15) and other laboratories (16-19) have shown that upon binding to its target cells hCG

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The abbreviation used is: hCG, human chorionic gonadotropin.

is subsequently internalized and degraded by receptor-mediated endocytosis. This pathway does not appear to be involved in the expression of the biological actions of hCG but may be important for other regulatory functions, such as the termination of hormone action (20) and receptor down regulation (21).

hCG is a complex glycoprotein composed of two different subunits (designated α and β) joined by noncovalent interactions (22, 23). The isolated subunits are biologically inactive but can be recombined to form a biologically active product (24). This provides the means to prepare radioactively labeled derivatives of the hormone in which the label is exclusively localized in one subunit and allows one to follow the fate of each hormone subunit (14, 15, 25-29). Recent reports from this (14, 15) and other laboratories (25-29) indicate that when these labeled derivatives are used to follow the fate of hCG, most tissues (target and nontarget) show a preferential retention of the radioactivity derived from the β subunit. These results suggest that at some point the hormone subunits are processed as different entities and raise the possibility that subunit dissociation may be involved in the biological actions of hCG and the other glycoprotein hormones (23, 25-28).

The experiments described herein were aimed at characterizing the fate of the two subunits of hCG when the hormone binds to cultured Leydig tumor cells. The results presented show that (a) upon binding to the surface receptor, hCG is internalized in the intact form and it reaches its place of degradation (presumably the lysosomes) in the intact form; and (b) the pathway for degradation of the internalized hormone is complex and explains the preferential retention of the radioactivity derived from the β subunit observed by us (14, 15) and others (25-29).

MATERIALS AND METHODS

Hormones and Cells—hCG and subunits were obtained from the National Institute of Child Health and Human Development. Radiiodinated derivatives of hCG labeled exclusively in the α (α-125I-hCG) or β subunit (β-125I-hCG) were prepared as previously described (14, 15). Crude hCG (3000 IU/ml) was obtained from Sigma.

All experiments were done using a clonal strain of Leydig tumor cells (designated MA-10) (30). Experimental cultures were used 3-4 days after plating.

Standard Assay Conditions and Acid Treatment—All assays were performed in 6-cm culture dishes containing 2-4 ml of Waymouth MB752/liter modified to contain 20 mM 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid, 1.12 g/liter of NaHCO3, 1 mg/ml of albumin, 40 μg/ml of gentamicin, pH 7.4 (assay medium). Prior to the assay, the growth medium was aspirated, and the dishes were washed twice with 2-ml portions of assay medium equilibrated at the appropriate temperature. For the 4°C experiments, the dishes were preincubated at 4°C for 30 min prior to the washing step. The binding of 125I-labeled hCG was always measured using three dishes. Two of them received 125I-labeled hCG only (total radioactivity), and the third also

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received (in addition to \(^{125}\)I-labeled hCG) 25 IU/ml of crude hCG (nonspecific radioactivity). The binding reaction was terminated by aspirating the medium and washing the dishes 5 times (2 ml each time) with ice-cold Hank's balanced salt solution containing 1 mg/ml of albumin.

The dishes then received 1.5 ml of ice-cold 50 mM glycine, 100 mM NaCl, pH 3.0. After 2 min at 4 °C, this solution was removed and the dishes were washed with 1 ml of the cold acid solution. The wash was combined with the original solution and counted (acid-sensitive radioactivity), and the cells were dissolved in 0.5 N NaOH and counted (acid resistant radioactivity). The cell extracts were subsequently used to determine DNA (30).

Under these conditions, the nonspecific radioactivity accounted for at most 5% of the total radioactivity. All data was corrected accordingly. The amount of radioactivity present is duplicate "total radioactivity" tubes and the amount of DNA/dish varied by at most 15%.

 Autoradiography—Cells were incubated in 2 ml of assay medium containing 20 ng/ml of \(^{125}\)I-hCG for 2 h at 37 or 4 °C. After washing to remove the free hormone, 1 ml of isotonic saline was added to each dish. The dishes were scraped with a rubber policeman, transferred to a tube, and centrifuged. The supernatant was aspirated, and the pellets were fixed for 1 h at 4 °C in 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.4. The glutaraldehyde was aspirated, and the pellets were washed with buffer and prepared for autoradiography as described by others (7, 31, 32). Samples were examined in a Hitachi HV-11B electron microscope operated at 75 kV. Photographs were taken at a magnification of about 10,000 and printed at a final magnification of 26,000. About 50 photographs of consecutive grains were counted (100-150 grains) on well preserved cells. The grain centers were determined by overlaying the grain with the smallest possible circle, and the distance from the center of the grain to the nearest plasma membrane was measured (7, 31, 32).

Analysis of Surface-bound and Internalized Radioactivity—Dishes were incubated with labeled hormone as described above. At the times indicated, they were washed with cold Hank's balanced salt solution without albumin. The surface-bound radioactivity was released (acid sensitive, regardless of the time of incubation. At 4 °C, most of the cell-bound radioactivity was acid sensitive only at early time points. Moreover, the time course of the accumulation of acid sensitive and acid-resistant radioactivity suggested a precursor-product relationship whereby the former becomes acid resistant as a function of time at 37 °C.

Conclusive proof for the hypothesis that the acid resistant radioactivity is internalized was obtained by autoradiography. Cells were incubated with \(^{125}\)I-hCG for 2 h at 37 or 4 °C, prepared for autoradiography, and the grain distribution was analyzed with respect to its distance to the nearest plasma membrane (1, 31, 32). The data presented in Fig. 2 show that at 4 °C, about 90% of the grains were localized at the plasma membrane, while at 37 °C, only about 60% of the grains were localized at the membrane. Acid treatment of parallel samples revealed that 91 and 59% of the cell-bound radioactivity was acid sensitive at 4 and 37 °C, respectively.

These results show that the acid treatment removes only the surface-bound hormone and leaves the cell morphologically and functionally intact. The acid treatment procedure described here also releases surface-bound epidermal growth factor with the same efficiency (Lloyd, C., and Ascoli, M., manuscript in preparation).
Steroid production by acid-treated cells

Dishes were incubated for 2 min (4°C) with medium or glycine/NaCl and washed with 1 ml of the appropriate solution and 2 ml of medium. Duplicate dishes were then incubated with 4 ml of medium containing buffer only (basal), 3.4 x 10^{-7} M hCG, or 1 x 10^{-9} M 8-Br-cAMP. Progesterone was determined after a 4-h incubation at 37°C as described elsewhere (30).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Basal</th>
<th>hCG</th>
<th>8-Br-cAMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.07</td>
<td>62</td>
<td>69</td>
</tr>
<tr>
<td>Glycine-NaCl</td>
<td>0.07</td>
<td>63</td>
<td>69</td>
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</tbody>
</table>

![Graph of progesterone/μg DNA × 4 h](image)

The data presented in Fig. 1 show that hCG is internalized as the intact hormone. The difference in the rate of degradation of α-

hCG and β-

hCG (cf. Fig. 3), however, shows that once internalized these derivatives are processed differently. These results could be due to differences in the mechanism and/or location of degradation or differences in the intrinsic properties of the two derivatives, such as susceptibility to proteolysis or location of the iodinated tyrosyl residues.

Both hormone derivatives appear to be degraded by the same mechanisms. Their final degradation products are identical, and the degradation of either derivative can be inhibited (to the same extent) with compounds that block lysosomal internalized hormone degraded. The data also show that α-

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hCG are internalized at the same rate but degraded at different rates.

The identity of the rates of internalization suggests that the hormone is internalized in the intact form (i.e., without dissociating into subunits). This hypothesis was confirmed by the following experiment. Cells were incubated with α-

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hCG as outlined above (cf. Fig. 3). The acid-sensitive radioactivity was collected at the end of the binding period (t = 0) and after half of the radioactivity had been internalized (t = 1 h) and analyzed by polyacrylamide gel electrophoresis. The data presented in Fig. 4 show that the acid-sensitive radioactivity is intact hCG, not individual subunits.

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respectively. The proteolytic activity present in these extracts was inhibited with leupeptin, p-chloromercuribenzoic acid (a sulfhydryl-blocking agent), and by increasing the pH to 7 (data not shown). These results suggest that the different rates of degradation of the internalized α-\(^{125}\)I-hCG and β-\(^{125}\)I-hCG are largely due to differences in their susceptibility to lysosomal proteolysis, rather than to differences in the mechanism and/or location of degradation.

One possible explanation for the observed differences is that once the intact hormone is internalized, it may dissociate into subunits, and the subunits degraded independently at different rates. Alternatively, the hormone may be degraded in the intact form. If this is the case, then the different rates of degradation may simply reflect differences in the location of the iodinated tyrosine residues. In order to differentiate between these possibilities, the following experiment was done. Cells were allowed to internalize and degrade α-\(^{125}\)I-hCG and β-\(^{125}\)I-hCG as outlined above (cf. Fig. 3). At different times, the surface-bound radioactivity was removed, and the internalized radioactivity was extracted and analyzed by electrophoresis. The internalized radioactivity was extracted under acidic conditions that favor the dissociation of the hormone from its receptor (cf. Table I), without dissociating the hormone into subunits (Figs. 6A and 7A).

The internalized radioactivity derived from α-\(^{125}\)I-hCG (Fig. 6, B–D) was resolved into three components with RF values of 0.48 (Peak α-I), 0.55 (Peak α-II), and 0.78 (Peak α-III). Peaks α-I and α-III co-migrate with intact hCG and the α subunit, respectively. Peak α-II appears to be degradation product of hCG (see below), and it increases with time, at the expense of Peak α-I. Also note that when the internalized radioactivity is boiled before electrophoresis (to dissociate the subunits) only one component that co-migrates with the intact α subunit (i.e. Peak α-III) is observed (Fig. 6E). This result shows that Peaks α-I and α-II contain intact α subunit.

In order to test if these hormone derivatives have different susceptibility to proteolysis, the following experiment was performed. α-\(^{125}\)I-hCG or β-\(^{125}\)I-hCG (256 ng/ml) was incubated with a cell-free extract in 0.1 M sodium acetate containing 1 mM ethylene diaminetraacetate and 2 mM dithiothreitol, pH 4 (38). After precipitation with 5% trichloroacetic acid, it was found that 23 ± 2% and 12 ± 1% of the added α-\(^{125}\)I-hCG and β-\(^{125}\)I-hCG had been degraded, respectively. The proteolytic activity present in these extracts seems to be due to lysosomal enzymes, because it can be inhibited with leupeptin, p-chloromercuribenzoic acid (a physiologic proteolytic inhibitors), and by increasing the pH to 7 (data not shown). These results suggest that the different rates of degradation of the internalized α-\(^{125}\)I-hCG and β-\(^{125}\)I-hCG are largely due to differences in their susceptibility to lysosomal proteolysis, rather than to differences in the mechanism and/or location of degradation.

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hCG Internalization and Degradation

The internalized radioactivity derived from $\beta^{125}$I-hCG (Fig. 7, B–D) was resolved into four components with $R_f$ values of 0.48 (Peak I), 0.55 (Peak II), 0.70 (Peak III), and >0.8 (Peak IV). Peak I co-migrates with intact hCG; Peak II has the same $R_f$ as the Peak observed with $\alpha^{125}$I-hCG (cf. Fig. 6), and it also increases with time at the expense of Peak I. Peaks III and IV appear to be degradation products of the $\beta$ subunit, since they are not present in the cells that internalize $\alpha^{125}$I-hCG (cf. Fig. 6), and they migrate faster than the native subunit. When the internalized radioactivity derived from $\beta^{125}$I-hCG was boiled before electrophoresis, three components were observed (Fig. 7E). The first peak co-migrates with the $\beta$ subunit; the second and third peaks correspond to Peaks III and IV described above. The distribution of radioactivity among these components suggests that Peak II contains a partially degraded $\beta$ subunit.

Based on these observations, it is concluded that the component with $R_f = 0.48$ (Peak I in Fig. 6, B–D, and B–I in Fig. 7, B–D) is intact hCG, and the component with $R_f = 0.55$ (Peak I in Fig. 6, B–D and $\beta$ II in Fig. 7, B–D) is composed of an intact $\alpha$ subunit and a partially degraded $\beta$ subunit.

**DISCUSSION**

Previous observations from this laboratory have shown that upon binding to Leydig tumor cells, both subunits of the receptor-bound hCG are ultimately degraded by a process whose biochemical properties closely resemble those of the receptor-mediated endocytosis (10–15). The experiments presented herein show new morphological and biochemical evidence that the process of hCG degradation occurs by this pathway. Thus, it is now reported that (a) hCG internalization can be visualized and quantitated by autoradiography (cf. Fig. 2); and (b) the surface-bound and internalized hormone can be easily distinguished by briefly exposing the cells to isotonic glycine at pH 3.0 (cf. Table I and Fig. 1).

Using the acid release method described in this report and two radioactively labeled derivatives of hCG labeled exclusively in the $\alpha$ or $\beta$ subunits, I was able to follow the fate of the hormone subunits during the internalization and degradation of the receptor-bound hormone. The results show that hCG is internalized in the intact form (i.e., without dissociating into subunits) and that it reaches its place of degradation (presumably the lysosomes) in the intact form.

The pathway for degradation of the internalized hormone is complex, and it appears to begin with the processing of one or both subunits of the intact hormone, resulting in a product that dissociates into subunits prior to further degradation (Figs. 6 and 7). The data presented suggest that the first degradation product ($R_f = 0.55$ in Figs. 6 and 7) is composed of an intact $\alpha$ subunit and a partially degraded $\beta$ subunit. This product then appears to dissociate into a free intact $\alpha$ subunit ($R_f = 0.78$ in Fig. 6) and a partially degraded $\beta$ subunit ($R_f = 0.70$ in Fig. 7). The dissociated subunits are then degraded independently. Further degradation of the $\alpha$ subunit occurs in such a way that the only detectable degradation product is extracellular monoiodotyrosine (Figs. 3 and 6). On the other hand, further degradation of the partially degraded $\beta$ subunit results in the formation of several intracellular products, as well as extracellular monoiodotyrosine (Figs. 3 and 7).

The apparent differences in the degradation of each subunit may be due to differences in the positions of the iodinated tyrosyl residues or intrinsic differences in their susceptibility to proteolysis. Regardless of the actual sequence of events involved in the process of degradation, the data presented clearly show that the slower release of monoiodotyrosine from $\beta^{125}$I-hCG is due to the formation of several labeled intracellular degradation products. These results provide an explanation for the preferential retention of radioactivity derived from the $\beta$ subunit previously reported by us (14, 15) and others (25–28).

Previous results from this laboratory have shown that NH$_4$Cl, chloroquine, and leupeptin effectively block the degradation of both hormone subunits without affecting the hormone-stimulated steroid production (10–15). Inasmuch as these compounds have now been shown to inhibit proteolysis of the internalized hormone (cf. Fig. 5), it is concluded that the degradation of the internalized hormone is not required for the stimulation of steroid biosynthesis. In other studies (not shown), when the intracellular radioactivity derived from NH$_4$Cl or leupeptin-treated cells was analyzed, it was found that NH$_4$Cl blocked the formation of all intracellular degradation products, while leupeptin allowed the formation of the first product ($R_f = 0.55$, see Figs. 6 and 7) to occur but blocked the formation of all the other products. Thus, it can also be concluded that hormone dissociation is not involved in the stimulation of steroidogenesis.

Thus, from the results presented herein and those published previously (14, 15, 20), it can now be concluded that hCG stimulates cAMP and steroid production while the hormone is bound to the cell surface. Since dissociation of the hormone into subunits does not occur at this stage, the hormone does not need to dissociate in order to stimulate cAMP and steroid production.

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hCG Internalization and Degradation