

Differential interactions of human choriogonadotropin and its antagonistic aglycosylated analog with their receptor

(receptor domain/affinity labeling)

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ABSTRACT Human choriogonadotropin (hCG) is a heterodimeric hormone consisting of an α subunit and a β subunit. hCG and aglycosylated hCG (aghCG) have similar receptor binding affinities but differ in their ability to activate hormone-responsive adenylate cyclase. aghCG is an effective antagonist. The mechanisms of this antagonism and interactions of antagonistic aghCG with the receptor are not understood. To address this critical question, we have examined the interaction of this hormone analog with the receptor. The hormone receptor on porcine granulosa cells is a glycoprotein of 86 kDa and has three domains of 24 kDa, 28 kDa, and 34 kDa, which are disulfide-linked. They undergo proteolysis, particularly when bound to the hormones, to produce three polypeptide components. These three receptor components can readily be identified through the use of affinity labeling with the hormones. Affinity labeling with an amino-specific homobifunctional reagent and subsequent cleavage indicate that hCG is cross-linked directly to the 24-kDa receptor component. In contrast, aghCG is cross-linked directly to the 34-kDa component. The peptide map of the cross-linked aghCG-34-kDa receptor component produced by papain treatment is different from the peptide map of the cross-linked complex of hCG-24-kDa component. This difference in receptor binding may be a factor determining the success or failure of signal transduction from the receptor to the effector system, guanine nucleotide-binding regulatory protein, and adenylate cyclase.

Pituitary lutropin and placental human choriogonadotropin (hCG) are heterodimeric glycoprotein hormones that recognize the same receptor and elicit similar physiological responses (1, 2). Carbohydrates of these hormones constitute up to 30% of the total mass and are N- and O-linked (3, 4). When oligosaccharides are removed, the resulting aglycosylated hCG (aghCG) becomes an antagonist, failing to activate adenylate cyclase but retaining its receptor-binding activity (5). It has been reported that carbohydrates of both subunits (6–8) and particularly those on the α subunit are critical for the biological activity of hCG (8–10). The antagonism of aghCG can be reversed by complexing receptor-bound aghCG with antibodies specific to the β subunit of aghCG (11) and, therefore, it is thought to be caused by incorrect interactions of aghCG with the receptor (12). To investigate the antagonistic interaction of aghCG, we have labeled the lutropin receptor of porcine granulosa cells with aghCG and hCG. The molecular mass of the porcine lutropin receptor is estimated to be 85–86 kDa (13, 14) and the polypeptide moiety alone is 75 kDa (13). The receptor readily undergoes proteolytic cleavage, particularly when bound to the hormone, to produce three separate polypeptide components of approximately 24 kDa, 28 kDa, and 34 kDa (15–17). These three components represent three distinct domains of the

receptor and can easily be identified when affinity labeled with the hormone (16). The 24-kDa and 34-kDa components are glycosylated with N-linked oligosaccharides and sialic acids (18) and are thought to be exposed to the cell exterior and to share hormone binding sites (15). When the receptor is affinity-labeled with hCG by the use of amino-specific homobifunctional reagents, the hormone is consistently cross-linked directly to the 24-kDa component but not to the 28-kDa and 34-kDa components. These two components are normally linked to the 24-kDa component by disulfide bonds in a linear fashion (16): the 24-kDa component is disulfide-linked to the 28-kDa component and the 28-kDa component is disulfide-linked to the 34-kDa component. In this communication we report that antagonistic aghCG is cross-linked to the 34-kDa component, instead of to the 24-kDa component.

EXPERIMENTAL PROCEDURES

hCG batch CR-123 was supplied by the Center for Population Research (National Institute of Child Health and Human Development). The biological potency was 12,780 international units/mg. aghCG was prepared as described (19) and the alkaline-cleavable reagent bis[2-(succinimidooxycarbonyloxy)ethyl]sulfone (SES or also known as BSOOES) was purchased from Pierce. Preparation of porcine granulosa cells, affinity labeling, cleavage of cross-linked complexes, and peptide mapping were done as described (18).

RESULTS AND DISCUSSION

Porcine granulosa cells were incubated with ^{125}I -labeled hCG and washed to remove unbound hormone. Nonspecific binding in the presence of 100 nM unlabeled hCG was 2–7% of total binding. When ^{125}I -labeled hCG, which was bound to the cells, was cross-linked with increasing concentrations of SES and electrophoresed under reducing conditions, three cross-linked hormone-receptor complexes were produced, in addition to the hormone bands (Fig. 1). To determine the apparent molecular masses of the complexes, cross-linked samples were electrophoresed on gels of various polyacrylamide concentrations: 7, 8, 9, 11, 6–12, 7–12, 8–12, 9–12, and 10–12%. Although the molecular mass estimates varied depending on the gradient and concentration of polyacrylamide, the average values were 74 kDa, 102 kDa, and 136 kDa in comparison with 50 kDa for the hCG $\alpha\beta$ dimer. These values are more accurate than molecular masses determined by a single gel system (14). Affinity labeling with aghCG produced cross-linked hormone-receptor complexes with apparent molecular masses of 72 kDa, 100 kDa, and 124 kDa whereas the aghCG $\alpha\beta$ dimer was 38 kDa. As expected, the sizes of cross-linked complexes differ depending on the hormone used (hCG versus aghCG).

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Abbreviations: hCG, human choriogonadotropin; aghCG, aglycosylated human choriogonadotropin; SES, bis[2-(succinimidooxycarbonyloxy)ethyl]sulfone.

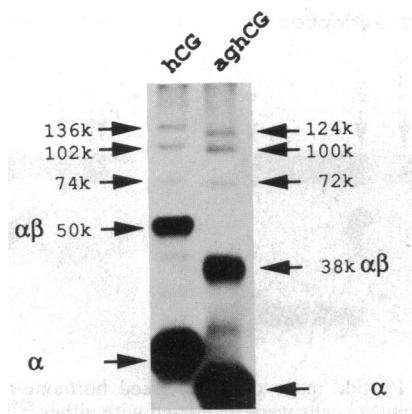


FIG. 1. Affinity labeling of the porcine lutropin receptor. Porcine granulosa cells were incubated with either ¹²⁵I-labeled hCG or ¹²⁵I-labeled aghCG (as indicated), washed, and treated with a homobifunctional reagent, SES. The cells were solubilized under reducing conditions and electrophoresed. The gel was exposed to x-ray film. The apparent molecular masses of the hormones and cross-linked hormone-receptor complexes were estimated on the basis of their electrophoretic mobilities on gels of various polyacrylamide concentrations, 7, 8, 9, 11, 6–12, 7–12, 8–12, 9–12, and 10–12%. k, kDa.

Fig. 2 shows that the extent of cross-linking of hCG to the receptor was dependent on the SES concentration. At low concentrations of SES, the 74-kDa complex was formed, and at higher concentrations the 102-kDa complex and the 136-kDa complex become apparent. To analyze the composition of these cross-linked hormone-receptor complexes, a first-dimension gel lane was excised, incubated in cleaving solution to partially cleave cross-links, placed on the top of a fresh slab gel, and electrophoresed to resolve cleaved components. The results are summarized in Table 1. In the second-dimension gel, the 136-kDa band produced the 102-kDa band, suggesting the loss of a 34-kDa component. The 102-kDa band produced the 74-kDa band, indicating the loss of a 28-kDa component. The 74-kDa band produced the 50-kDa hormone $\alpha\beta$ dimer band, showing the release of a 24-kDa component.

These results demonstrate that the 74-kDa complex consists of the hormone $\alpha\beta$ dimer and a component of 24 kDa (74 kDa = 50 kDa + 24 kDa), and the 102-kDa complex consists of this 74-kDa complex and an additional 28-kDa component (102 kDa = 74 kDa + 28 kDa). Likewise, the 136-kDa complex is composed of the 102-kDa complex and an additional 34-kDa component. The sum of 24 kDa, 28 kDa, plus 34 kDa is 86 kDa, which represents a hormone binding unit

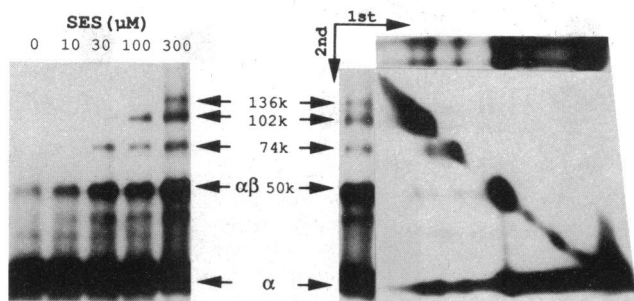


FIG. 2. Cross-linking and cleaving of ¹²⁵I-labeled hCG-receptor complexes under reducing conditions. ¹²⁵I-labeled hCG-complexed cells were treated with increasing concentrations of SES (in μ M) and solubilized under reducing conditions. After electrophoresis a first-dimension gel lane was cut out, treated in alkaline solution to cleave cross-links, and subjected to second-dimension electrophoresis. k, kDa.

Table 1. Composition of cross-linked complexes

Complex	First-dimension component, kDa	Second-dimension components
hCG-R	50	hCG $\alpha\beta$
	74	hCG $\alpha\beta$, 24 kDa
	102	hCG $\alpha\beta$, 24 kDa, 28 kDa
	136	hCG $\alpha\beta$, 24 kDa, 28 kDa, 34 kDa
aghCG-R	38	aghCG $\alpha\beta$
	72	aghCG $\alpha\beta$, 34 kDa
	100	aghCG $\alpha\beta$, 34 kDa, 28 kDa
	124	aghCG $\alpha\beta$, 34 kDa, 28 kDa, 24 kDa

Cross-linked hCG-receptor complexes (hCG-R) or aghCG-receptor complexes (aghCG-R), which were resolved in a first-dimension gel, were treated in alkaline solution to cleave cross-links, and subjected to second-dimension electrophoresis. Components released from each cross-linked complex were determined as shown in Figs. 1 and 4.

of the receptor (15). This value is in agreement with a report of 85 kDa for purified and cloned porcine lutropin receptor (13). In intact receptors, the 24-kDa, the 28-kDa, and the 34-kDa components probably represent distinct domains (15).

Affinity-labeled hCG-receptor complexes appear as a single major band of 136 kDa under nonreducing conditions (Fig. 3). To examine the effect of reduction on this unreduced hormone-receptor complex, a gel of the unreduced affinity-labeled sample was excised, treated with dithiothreitol, and analyzed on a fresh slab gel (Fig. 3). From the 136-kDa complex, the 102-kDa and 74-kDa bands were produced, suggesting the release of the 34-kDa component or both the 34-kDa and the 28-kDa components, respectively. Since no additional bands were released from the 74-kDa complex under reducing conditions, its constituents (the 24-kDa receptor component and the 50-kDa hCG $\alpha\beta$ dimer) are likely to be covalently linked by SES through bonds other than disulfides. Therefore, the hormone appears to be first cross-linked directly to the 24-kDa component, the 24-kDa component is disulfide-linked to the 28-kDa component, and the 28-kDa component is disulfide-linked to the 34-kDa component. This conclusion is in complete agreement with the previous prediction of disulfide bonds in the lutropin receptor (16, 20). The hCG $\alpha\beta$ dimer after SES cross-linking appears as two species on gels, one as the (upper) reduced form and the other as the (lower) unreduced form as reported (16). The unreduced form was converted to the reduced form on the second-dimension reducing gel (Fig. 3).

Affinity labeling of the lutropin receptor with aghCG was also dependent on SES concentration and revealed a similar trend of the incremental formation of three cross-linked

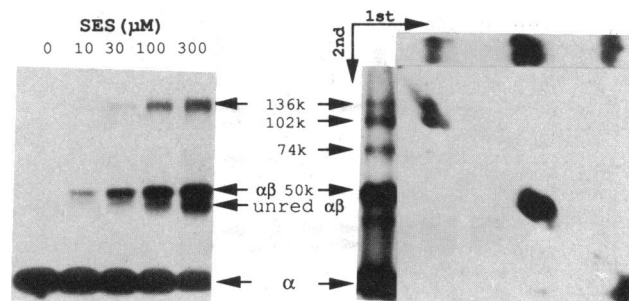


FIG. 3. Cross-linking and cleaving of ¹²⁵I-labeled hCG-receptor complexes under nonreducing conditions. ¹²⁵I-labeled hCG-complexed cells were treated with increasing concentrations of SES (in μ M) and solubilized under nonreducing conditions. After electrophoresis a first-dimension gel lane was cut out, treated in 100 mM dithiothreitol to cleave disulfide bonds, and subjected to second-dimension electrophoresis. k, kDa.

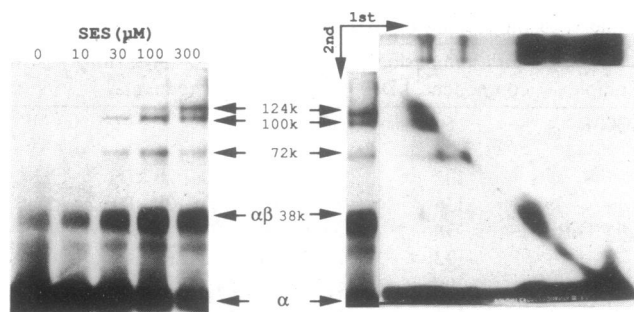


FIG. 4. Cross-linking and cleaving of ^{125}I -labeled aghCG-receptor complexes under reducing conditions. ^{125}I -labeled aghCG-complexed cells were treated with increasing concentrations of SES (in μM) and solubilized under reducing conditions. After electrophoresis a first-dimension gel lane was cut out, treated in alkaline solution to cleave cross-links, and subjected to second-dimension electrophoresis. k, kDa.

hormone-receptor complexes, with the smaller complex being formed first, followed by the formation of the larger complexes (Fig. 4). The appearance of the largest 124-kDa complex coincides with the diminution of the smallest 72-kDa complex and, therefore, suggests a precursor-product relationship. Partial alkaline cleavage of the 124-kDa cross-linked complex produced the 100-kDa complex, indicating the loss of a 24-kDa component. Similarly the 100-kDa complex produced the 72-kDa complex by releasing a 28-kDa component, and the 72-kDa complex produced the 38-kDa aghCG $\alpha\beta$ dimer upon release of a 34-kDa component (Table 1).

Under nonreducing conditions, only the 124-kDa band of cross-linked aghCG-receptor complex appeared (Fig. 5). However, upon reduction, the 100-kDa and 72-kDa complexes were produced from the 124-kDa complex, indicating the release of either the 24-kDa component or both the 24-kDa and 28-kDa components, respectively. The 72-kDa complex was resistant to reduction-dependent cleavage, suggesting that its two components, the 34-kDa receptor component and the 38-kDa aghCG $\alpha\beta$ dimer, are linked by the SES cross-linker but not by disulfide bonds. These results indicate that aghCG is cross-linked by SES directly to the 34-kDa receptor component. This conclusion is consistent with the observation that both the aghCG α subunit and the aghCG $\alpha\beta$ dimer were released when the 124-kDa complex was subjected to partial alkaline cleavage (Fig. 5). Therefore, it is reasonable to conclude that aghCG is cross-linked to the

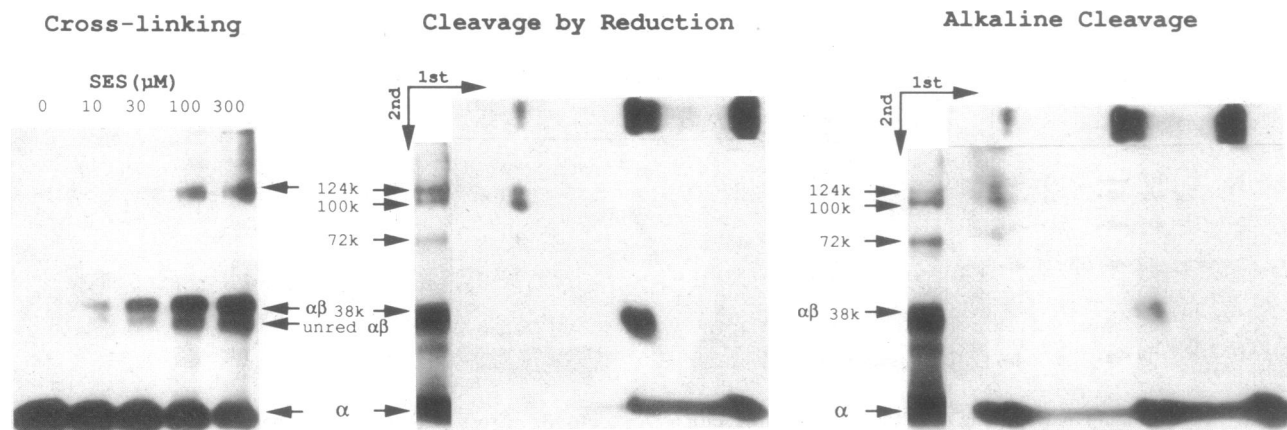


FIG. 5. Cross-linking and cleaving of ^{125}I -labeled aghCG-receptor complexes under nonreducing conditions. ^{125}I -labeled aghCG-complexed cells were treated with increasing concentrations of SES (in μM), solubilized under nonreducing conditions, and electrophoresed (Left). After electrophoresis a first-dimension gel lane was cut out, treated in 100 mM dithiothreitol to cleave disulfide bonds, and subjected to second-dimension electrophoresis (Center). Another first-dimension gel lane was cut out, treated in alkaline solution to cleave cross-links and to reduce disulfide bonds, and subjected to second-dimension electrophoresis (Right). k, kDa.

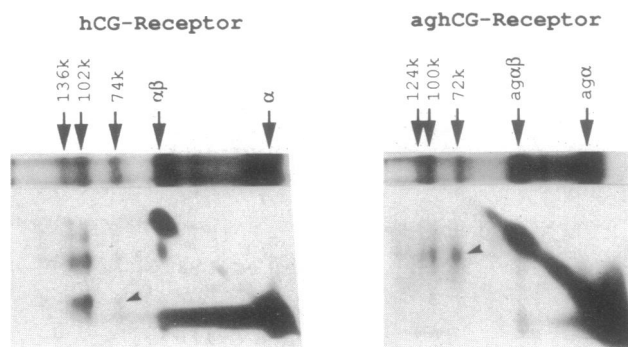


FIG. 6. Peptide maps of cross-linked hormone-receptor complexes. Granulosa cells were incubated with either ^{125}I -labeled hCG or ^{125}I -labeled aghCG, washed, and treated with SES. The cells were solubilized under reducing conditions and electrophoresed. A first-dimension gel lane of each sample was cut out, treated with papain to digest proteins, and subjected to second-dimension electrophoresis. Arrow heads indicate the smallest fragment of the hCG-receptor complexes and the aghCG-receptor complexes. k, kDa.

34-kDa component, which is disulfide-linked to the 28-kDa and 24-kDa components.

Although these three released components of 34 kDa, 28 kDa, and 24 kDa are equal in size to the released components from the cross-linked hCG-receptor complexes, the sequence of the cleavage-dependent release is reversed (Table 1). Furthermore, the receptor components directly cross-linked to hCG and aghCG are not the same, with hCG being cross-linked to the 24-kDa component whereas aghCG is cross-linked to the 34-kDa component. These results indicate a significant difference in the interactions of the gonadotropin receptor with hCG and aghCG.

To further verify the chemical difference in the 74-kDa hCG-receptor complex and the 72-kDa aghCG-receptor complex, affinity-labeled hormone-receptor complexes that were resolved in a first-dimension gel were subjected to proteolytic digestion with papain, and the products were separated in a second-dimension gel (Fig. 6). It should be emphasized that only the hormone was radioactively labeled. Therefore, all the visualized radioactive fragments contain the hormone and, possibly, part of the receptor. It is striking to see that the smallest radiolabeled peptide produced from the 72-kDa aghCG-receptor complex migrated slower than the smallest fragment of the 74-kDa hCG-receptor complex. Since aghCG is smaller than hCG and the smallest fragment of the 72-kDa

complex (aghCG-34-kDa component) is larger than the smallest fragment of the 74-kDa complex (hCG-24-kDa component), the origin of the smallest fragments of these two complexes could not be same. The fact that the papain fragment of the 72-kDa, the 100-kDa, and the 124-kDa aghCG-receptor complexes is identical supports the view that the aghCG $\alpha\beta$ dimer is cross-linked directly to the 34-kDa component whereas the 28-kDa and the 24-kDa components are cross-linked to the 34 kDa component but not directly to the aghCG $\alpha\beta$ dimer. Likewise, peptide maps of hCG-receptor complexes are similar, indicating the direct cross-linking of the hCG $\alpha\beta$ dimer to the 24-kDa component but not to the 28-kDa and the 34-kDa components. One may argue that aghCG might be more resistant to papain than is hCG and, as a result, aghCG and its cross-linked complexes could not have been digested to produce fragments as small as those of hCG-receptor complexes. This argument, however, is not consistent with the fact that the aghCG $\alpha\beta$ dimer was digested by papain to smaller fragments than was the hCG $\alpha\beta$ dimer (Fig. 6) and that aghCG and other aglycosylated gonadotropins are more susceptible to proteolysis and have substantially shorter *in vivo* half-lives than intact hormones (21, 22).

hCG and aghCG bind the receptor differently. This difference can be better defined as we know more about the structure of the receptor and its hormone binding sites. The results of affinity labeling in comparison with the amino acid sequence of the receptor and its probable orientation in the cell membrane, which have been deduced from cDNA clones (13), are revealing. The cloned cDNA sequence suggests six potential N-linked oligosaccharide chains in the N-terminal half of the receptor. They are divided equally into two groups; the first three are located within the peptide domain₁₀₀₋₂₀₀ and the next three clustered in the peptide domain₃₀₁₋₃₁₅. In the 100-residue peptide between these two groups of oligosaccharides, there are a number of potential proteolysis sites including 11 trypsin sites, 2 of these being Lys-Lys. Cleavage at one of these points will certainly produce a glycopeptide similar to the 24-kDa component or the 34-kDa component. This cleavage will separate the two groups of N-linked oligosaccharides into two distinct glycopeptide fragments similar to the 24-kDa component and the 34-kDa component. On the other hand, the C-terminal half includes seven transmembrane domains and has no N-linked oligosaccharides. This is impressively consistent with previous affinity-labeling results demonstrating that the 24-kDa and the 34-kDa receptor components are glycosylated to a similar extent with N-linked oligosaccharides and sialic acids and are exposed to the cell exterior, in contrast to the 28-kDa component, which is not glycosylated (15, 18).

Therefore, the 28-kDa component appears to be the C terminus. Between the remaining 24-kDa and 34-kDa components, one is the N terminus and the other represents the mid-section of the receptor. There is evidence suggesting that the hormone binding site in the receptor is composed of several epitopes belonging to the three domains (15, 23). Likewise, the receptor binding sites in the hormone consists of several epitopes belonging to both subunits of the hormone (15, 23). Since both the 24-kDa and the 34-kDa components contribute parts of the hormone binding site, these two domains probably come into close contact with the hormone. The disulfide linkages between the three receptor domains do not appear to be disrupted regardless of hCG or aghCG binding. This result suggests that the difference in receptor binding of hCG and aghCG appears to be caused by subtle structural changes in the hormone and the receptor. The mutually exclusive cross-linking of hCG to the 24-kDa component or aghCG to the 34-kDa component indicates that the peptide epitopes at the hormone-receptor interface are sterically rigid. Under such conditions, the presumptive subtle changes upon aghCG binding to the 34-kDa domain is appar-

ently transduced to the 24-kDa domain and, as a result, the 24-kDa domain could not be cross-linked to aghCG. A similar type of the transduction of subtle conformational changes was observed when antibodies specific to the aghCG β subunit interacted with receptor-bound aghCG. This antibody binding reversed the antagonism of receptor-bound aghCG (11). How such subtle conformational changes dictate the fate of the signal transduction from the hormone to the receptor, then to guanine nucleotide-binding regulatory (G) protein is a mystery. Whether it involves the physical uncoupling of G protein from the receptor or is limited to minor conformational changes is yet to be determined.

Since the susceptibility of sialic acids in the 24-kDa and the 34-kDa components to neuraminidase varies depending on hormone binding (17), some of the carbohydrates are near the hormone binding sites and the cross-linking points at the hormone-receptor interface. Chemical cross-linking with SES requires pairs of free amines, one belonging to the hormone and the other belonging to the receptor. In fact, a number of lysines are present around the N-glycosylation sites. Whether any of these lysines of the receptor are involved in cross-linking either to hCG or to aghCG can be determined. It is clear that the lysines of the receptor cross-linked to hCG are not all the same as those cross-linked to aghCG. In addition to these differences in receptor binding, aghCG is known to label an 8-kDa polypeptide under certain conditions, particularly when the receptor is photo-affinity labeled (24). Whether this peptide corresponds to the 8-kDa γ subunit of G protein and whether the labeling occurs during internalization of the aghCG-receptor complexes are unknown.

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