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Structural Homologies in the Lutropin/Human Choriogonadotropin Receptor and the Follitropin Receptor on Porcine Granulosa Cells†

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ABSTRACT: In order to examine the structure of the human choriogonadotropin (hCG) receptor and the follitropin (FSH) receptor on porcine granulosa cells, the hormone receptors were photoaffinity-labeled or affinity-cross-linked. The resulting hormone–receptor complexes were analyzed by alkaline cleavage of cross-links, reduction of disulfides, and peptide maps. The results revealed striking similarities in the structure of the hormone receptors. Both appear to be oligomeric; the hCG receptor has at least four components of 18, 24, 28, and 34 kDa, whereas the FSH receptor shows three distinct components of 18, 22, and 34 kDa. The 24- and the 22-kDa components are the sites for the primary photoaffinity labeling or affinity cross-linking by hCG and FSH, respectively. These components were linked by intercomponent disulfides. Reduction of cross-linked complexes revealed that in the hCG receptor the 24-, the 28-, and the 34-kDa components were disulfide-linked sequentially in a linear form as were the 22-, the 18-, and the 34-kDa components in the FSH receptor. The peptide maps of cross-linked hCG–receptor and FSH–receptor complexes, however, were distinct, indicating that the hCG receptor and the FSH receptor were not identical.

Follitropin (FSH)[1] and lutropin (LH) are pituitary glycoprotein hormones and have distinct receptors on ovarian and testicular cells. The placental glycoprotein hormone hCG binds to the LH receptor and elicits similar physiological responses (Ward, 1978; Pierce & Parson, 1981). The structure of these three hormones are quite similar. They consist of two noncovalently associated, dissimilar subunits designated α and β. Within the same mammalian species, the α subunits of these hormones have virtually identical amino acid sequences.

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[1] Abbreviations: FSH, follitropin; LH, lutropin; hCG, human choriogonadotropin; TSH, thyrotropin; PBS, 0.15 M sodium chloride and 10 mM sodium phosphate (pH 7.4); NHS-ABG, *N*-hydroxysuccinimide ester of (4-azidobenzoyl)glycine; ABG-^{125}I-FSH, (4-azidobenzoyl)-glycyl-substituted ^{125}I-labeled follitropin; ABG-^{125}I-hCG, (4-azidobenzoyl)glycyl-substituted ^{125}I-labeled human choriogonadotropin; SES, bis[2-[[(succinimidooxy)carbonyl]oxy]ethyl] sulfone; NEM, *N*-ethylmaleimide; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

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In contrast, the β subunits have distinct sequences. In addition to these structural homologies, considerable similarities exist in the physiological responses elicited (Erickson, 1983). Since receptor binding is crucial for successful hormone action and ovarian granulosa cells carry receptors for both FSH and LH/hCG, there exists a potential for homology in the receptor structures. Since the precise chemical structures of the receptors are not known (Roche & Ryan, 1985) and in order to investigate the possible receptor homology, we have undertaken the protein chemical analysis of these receptors by photoaffinity labeling, affinity cross-linking, peptide mapping, and cleavage and reduction of covalently cross-linked hormone–receptor complexes.

Experimental Procedures

Materials. Human FSH (AFP4161B) and hCG (CR-123) were supplied by the Center for Population Research (National Institutes of Child Health and Human Development). Bovine TSH was a gift from Dr. John Pierce. The biological potency of FSH and hCG was 3925 IU/mg and 12 780 IU/mg, respectively. NHS-ABG was synthesized, and ABG-^{125}I-FSH and ABG-^{125}I-hCG were prepared as described (Ji & Ji, 1981). SES, an alkaline-cleavable homobifunctional reagent, was synthesized as described (Tesser et al., 1975). FSH and hCG were radioiodinated as described (Ji et al., 1981).

Cell Preparation. Porcine ovaries rich with either large follicles or small follicles but free of corpora lutea were selected and rinsed with 5 mM *N*-ethylmaleimide in PBS. Follicles were excised and granulosa cells collected in 80 mL of the buffer. It is important to prevent aggregation of cells by occasional, gentle mixing and by maintaining the cell concentration below 3×10^6 cells/mL and the temperature below 4 °C. Cells were collected by centrifugation at 600*g* for 10 min and rinsed twice in 15 mL of the buffer. All operations were carried out on ice or at 4 °C.

Photoaffinity Labeling. Granulosa cells isolated from large follicles were incubated with ABG-^{125}I-hCG or those isolated from small follicles with ABG-^{125}I-FSH for 60 min at 37 °C with constant, gentle shaking. Hormone-complexed cells, approximately 7×10^6 cells per sample, were washed twice, irradiated with a shortwave UV lamp (Mineralight Model R-52), and centrifuged. The cell pellet was suspended in 20–60 μL of the solubilizing solution containing 3% sodium dodecyl sulfate, 5% glycerol, and 100 mM dithiothreitol. After being boiled for 2 min, the suspension was centrifuged in a Beckman Ti 50.2 rotor at 40 000 rpm for 60 min at 20 °C. The supernatant was collected without disturbing the pellet and electrophoresed on a 9–12% gradient gel containing 9 M urea (Ji et al., 1985). Molecular weight marker proteins were soybean trypsin inhibitor, carbonic anhydrase, ovalbumin, bovine serum albumin, phosphorylase *b*, β-galactosidase, and myosin. Autoradiographs were prepared by exposing dried gels to X-ray film for 3–7 days at −30 °C.

Cross-Linking. Washed granulosa cells isolated from large follicles, 7×10^6 cells/400 μL of buffer, were incubated with ^{125}I-hCG at 37 °C for 60 min with constant, gentle shaking. Cells isolated from small follicles were incubated with ^{125}I-FSH. After incubation, cells were rinsed twice in 1 mL of PBS. The cell pellet was resuspended in 400 μL of PBS containing SES, incubated for 15 min at 25 °C, rinsed once in 50 mM glycine in PBS, solubilized, and electrophoresed as described above. To cleave cross-linked complexes resolved on first-dimension gels, gel lanes were cut and incubated for 90 min at 37 °C in the cleaving solution containing 0.1% sodium dodecyl sulfate, 10 mM dithiothreitol, and 100 mM Na_3PO_4 (pH 11.5). The treated gel lanes were mounted on the top of fresh gels and subjected to a second electrophoresis (Shin & Ji, 1985).

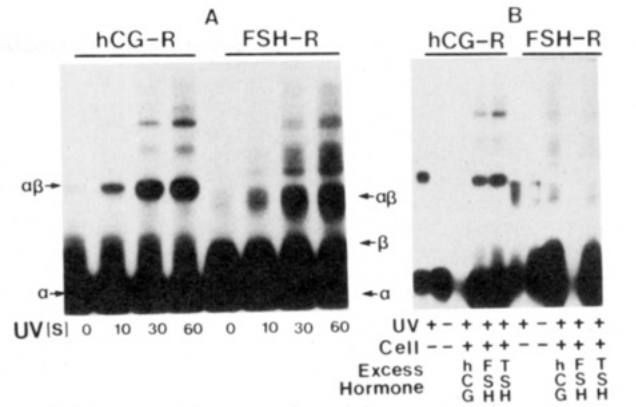

FIGURE 1: Photoaffinity labeling of the hCG receptor and the FSH receptor. (Panel A) Cells complexed in the presence of 5 mM *N*-ethylmaleimide with either ABG-^{125}I-hCG or ABG-^{125}I-FSH were irradiated with UV for varying periods, solubilized, and electrophoresed. (Panel B) Granulosa cells were incubated with either ABG-^{125}I-hCG (hCG-R) or ABG-^{125}I-FSH (FSH-R) in the presence of an excess of untreated hCG, FSH, or TSH, washed, photolyzed for 30 s, and electrophoresed. In addition, unbound ABG-^{125}I-hCG or ABG-^{125}I-FSH was electrophoresed with or without prior photolysis for 30 s.

Peptide Maps. Cross-linked samples were resolved on a first-dimensional gel. Gel lanes were excised and incubated in 50 mL of 125 mM Tris (pH 6.8) and 0.1% sodium dodecyl sulfate for 40 min at 25 °C. Following equilibration, the gel lanes were rinsed with distilled water and mounted on the top of fresh slab gels. A 4% stacking gel was cast up and around the first-dimensional gel lane and overlaid with 0.8 mL of 0.5% sodium dodecyl sulfate, 8% glycerol, pyronin Y, and 100 μg/mL either of *Staphylococcus aureus* V8 protease or papain. The gels were initially electrophoresed at 50 V for 30–60 min until the dye front reached three-fourths of the stacking gel. Electrophoresis was temporarily stopped for a 60-min incubation at 25 °C and then resumed at 100 V.

Results

Photoaffinity Labeling. The α and β subunits of ABG-^{125}I-FSH or ABG-^{125}I-hCG were separated by gel electrophoresis (Ji & Ji, 1981; Shin & Ji, 1985). The positions of the subunits of the two hormone derivatives were similar. The radioactive FSH β subunit showed a slower electrophoretic mobility than the α subunits (Figure 1). In contrast to successful radioiodination of FSH β, the radioactivity being 29% of the total FSH, hCG β was not radioiodinated as expected (Rebois, 1982).

Upon photolysis, the $\alpha\beta$ dimer bands of FSH and hCG increased, suggesting the successful derivatization and UV-dependent cross-linking of the hormones. The FSH $\alpha\beta$ dimer band position was considerably lower than that of the hCG $\alpha\beta$ dimer band. This different electrophoretic mobility is likely to be due to the faster electrophoretic mobility of FSH β than that of hCG β (Ji & Ji, 1981).

When granulosa cells were incubated with the hormone derivatives and the hormone-complexed cells were irradiated with UV, the photolyzed samples revealed several distinct additional bands of slower electrophoretic mobility (Figure 1, panel A). These bands became more intense as UV irradiation increased. Production of these bands was prevented by the presence of 50 nM of the respective native hormones but not by an excess amount of other hormones. It is evident that production of the bands requires the hormone derivatives specifically bound to granulosa cells and photolysis. This

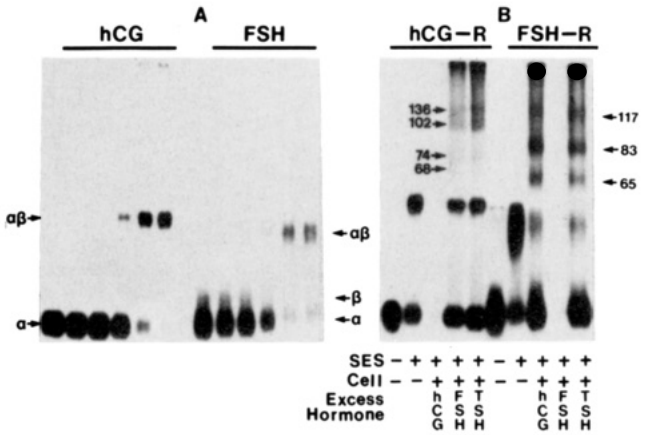

FIGURE 2: Cross-linking of hormones to the receptors. (Panel A) In the absence of cells, ^{125}I-hCG or ^{125}I-FSH was treated with increasing concentrations of SES (mM): 0, 0.03, 0.1, 0.3, 1, and 3. (Panel B) Granulosa cells were incubated with either ^{125}I-hCG (hCG-R) or ^{125}I-FSH (FSH-R) in the presence of an excess of hCG, FSH, or TSH and cross-linked with 1 mM SES. ^{125}I-Labeled hormones in the absence of the cells were shown with or without cross-linking with 1 mM SES.

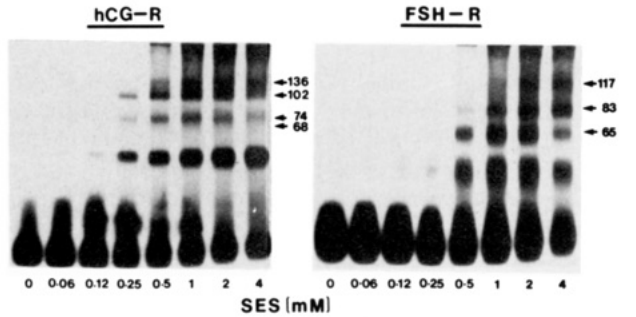

FIGURE 3: Reagent concentration dependent cross-linking. The cells complexed with ^{125}I-hCG or ^{125}I-FSH were cross-linked with increasing concentrations of SES.

suggests that the bands represent complexes of the hormone derivatives cross-linked to cellular components related to the respective hormone receptors.

Affinity Cross-Linking of hCG Receptor with ^{125}I-hCG and FSH Receptor with ^{125}I-FSH. When ^{125}I-labeled hormone-complexed cells were treated with an alkaline-cleavable homobifunctional reagent, SES, results similar to those of photoaffinity labeling were obtained (Figure 2), suggesting specific labeling with ^{125}I-hCG or ^{125}I-FSH of cellular components related to the hormone receptors. Formation of the high molecular weight complex bands was dependent on the SES concentration (Figure 3). When isolated granulosa cell membranes, instead of cells, were used for cross-linking, the results were identical, indicating the membrane origin of components associated in the higher molecular weight cross-linked complexes. Their apparent molecular weights were 136, 102, 74, and 68 kDa for ^{125}I-hCG–receptor complexes and 117, 83, and 65 kDa for ^{125}I-FSH–receptor complexes (Table I).

Composition of Cross-Linked Complexes. To investigate the composition of cross-linked hormone–receptor complexes, gel lanes of cross-linked samples were treated in the cleaving solution, laid on the top of fresh slab gels, and electrophoresed. As seen in the two-dimensional autoradiograph (Figure 4), cleaved components under the partial cleavage condition were seen as spots off the diagonal axis: In the case of ^{125}I-hCG–receptor complexes, the 102-, 74-kDa components and hCG $\alpha\beta$ were released from the 136-kDa complex, the 74-kDa component and hCG $\alpha\beta$ from the 102-kDa complex, and the hCG $\alpha\beta$ dimer from the 74-kDa component. These results

Table I: Cross-Linked Hormone–Receptor Complexes[a]

^{125}I-hCG–receptor (kDa)	^{125}I-FSH–receptor (kDa)
136	
102	117
74	83
68	65

[a] In order to determine the apparent molecular weights of the complexes, cross-linked samples were electrophoresed on reducing gels of varying acrylamide concentration, 7, 8, 9, 11, 6–12, 8–12, 9–12, and 10–12%. Although the molecular weight estimates varied depending on the gradient and concentration of acrylamide, the average values were presented above, relative to the molecular weight marker proteins. The $\alpha\beta$ dimer band corresponded to 50 kDa for hCG and 43 kDa for FSH. Accuracies are dependent on a variety of technical factors, and these molecular weights should, therefore, be considered tentative and be used primarily as designations for the band (polypeptides) identifications.

Table II: Composition of Cross-Linked Complexes[a]

^{125}I-hCG–receptor (kDa)	components (kDa)	^{125}I-FSH–receptor (kDa)	components (kDa)
68	hCG $\alpha\beta$, 18		
74	hCG $\alpha\beta$, 24	65	FSH $\alpha\beta$, 22
102	hCG $\alpha\beta$, 24, 28	83	FSH $\alpha\beta$, 22, 18
136	hCG $\alpha\beta$, 24, 28, 34	117	FSH $\alpha\beta$, 22, 18, 34

[a] The data were derived from the results in Figure 4 and Table I. The apparent molecular weights of the hCG $\alpha\beta$ and FSH $\alpha\beta$ dimers, 50 000 and 43 000, respectively, were used in the calculation.

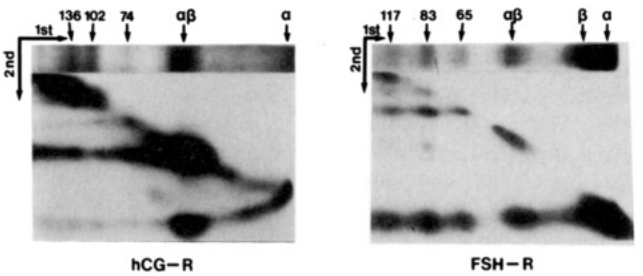

FIGURE 4: Cleavage of cross-links. Cross-linked ^{125}I-hCG–receptor or ^{125}I-FSH–receptor was resolved in the first-dimension gel, and the gel lanes were cut and treated for alkaline cleavage of cross-links. The treated gel lanes were mounted on the top of fresh slab gels and electrophoresed.

demonstrate that the 74-kDa complex is composed of the hCG $\alpha\beta$ dimer and a 24-kDa component (74 kDa = 50 kDa + 24 kDa), the 102-kDa complex of the 74-kDa complex and an additional 28-kDa component (102 kDa 74 kDa + 28 kDa = 50 kDa + 24 kDa + 28 kDa), and the 136-kDa complex of 102-kDa complex and an additional 34-kDa component (136 kDa = 102 kDa + 34 kDa = 74 kDa + 28 kDa + 34 kDa = 50 kDa + 24 kDa + 28 kDa + 34 kDa) as shown in Table II. The 68-kDa complex is composed of an 18-kDa component and the hCG $\alpha\beta$ dimer (68 kDa = 50 kDa + 18 kDa). In this calculation, the apparent molecular weight of the hCG $\alpha\beta$ dimer, 50 000, was used.

When cross-linked ^{125}I-FSH–receptor complexes were cleaved, the 83- and 65-kDa components and the FSH $\alpha\beta$ spots were released from the 117-kDa complex, the 65-kDa component and the $\alpha\beta$ dimer from the 83-kDa complex, and the $\alpha\beta$ dimer from the 65-kDa complex. These results demonstrate that the 65-kDa complex is composed of the FSH $\alpha\beta$ dimer and a 22-kDa component (65 kDa = 43 kDa + 22 kDa), the 83-kDa complex of the 65-kDa component and an additional 18-kDa component (83 kDa = 65 kDa + 18 kDa = 43 kDa + 22 kDa + 18 kDa), and the 117-kDa complex of the 83-kDa component and another component of 34 kDa (117 kDa = 83

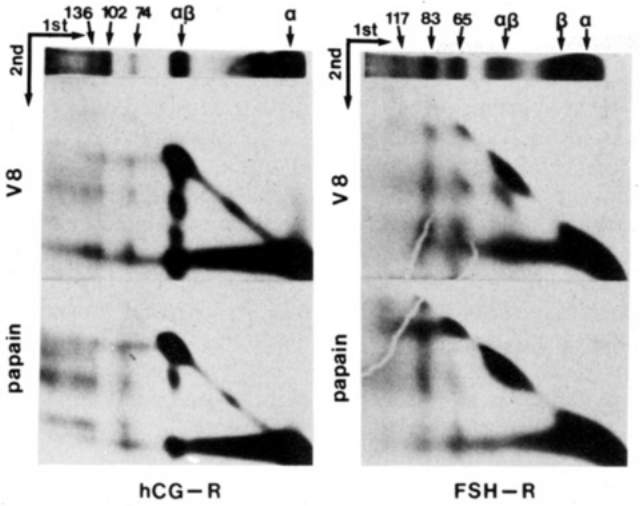

FIGURE 5: Peptide maps of cross-linked hormone–receptor complexes. The first-dimension gels of cross-linked ^{125}I-hCG–receptor or ^{125}I-FSH–receptor complexes were treated with either V8 or papain and electrophoresed on fresh slab gels (Bordieu & Crettol-Jarvineu, 1979).

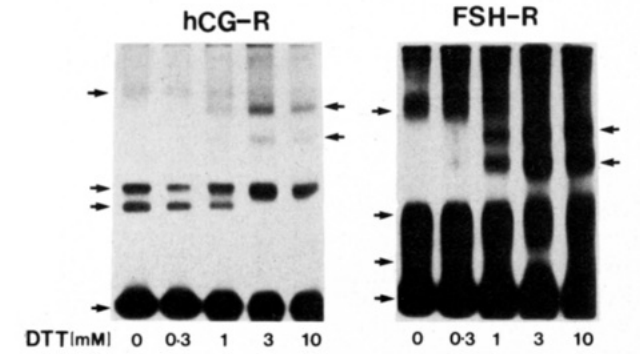

FIGURE 6: Reduction of disulfides in cross-linked hormone–receptor complexes. Cross-linked hormone–receptor complexes were solubilized in the presence of increasing concentrations of dithiothreitol (DTT) and electrophoresed.

kDa + 34 kDa = 65 kDa + 18 kDa + 34 kDa = 43 kDa + 22 kDa + 18 kDa + 34 kDa). The apparent molecular weight of the FSH $\alpha\beta$ dimer, 43 000, was used in this calculation. The cross-linked sample that was not cleaved did not show any of the released components.

Peptide Maps of Cross-Linked Complexes. First-dimensional gel lanes were cut and placed on the top of second-dimensional slab gels, overlaid with proteases, and electrophoresed (Bordier & Crettol-Jarvineu, 1979). As shown in Figure 5, the cross-linked ^{125}I-hCG–receptor complexes were degraded by V8. All of the 74-, 102-, and 136-kDa complexes broke down, eventually to similar peptide fragments. Treatments with papain produced peptide maps similar to but not identical with those of V8. These V8 and papain peptide maps of the cross-linked ^{125}I-hCG–receptor complexes were distinct from those of the cross-linked hCG $\alpha\beta$ dimer. ^{125}I-hCG α was resistant to the enzymes under the experimental condition.

V8 and papain peptide maps of the cross-linked ^{125}I-FSH–receptor complexes were different from those of the cross-linked ^{125}I-hCG–receptor complexes and the cross-linked FSH $\alpha\beta$ dimer. V8 peptide maps of the 117-, 83-, and 65-kDa complexes were similar, and so were the papain peptide maps.

Disulfide Linkages. Cross-linked hormone–receptor complexes were solubilized with varying concentrations of dithiothreitol and electrophoresed. In the absence of the reducing agent, both ^{125}I-hCG–receptor and ^{125}I-FSH–receptor complexes produced the high molecular weight complex bands (Figure 6). Under nonreducing conditions and in the absence

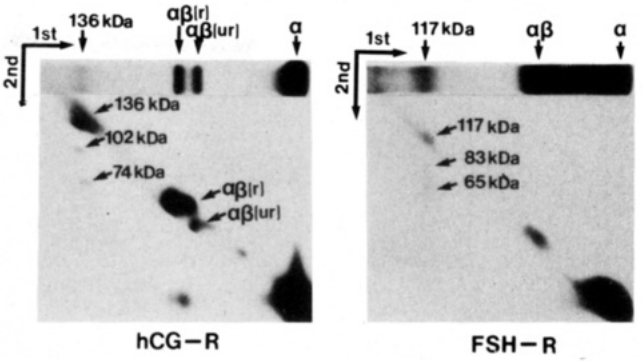

FIGURE 7: Two-dimensional analysis of reduction-dependent cleavage of cross-linked hormone–receptor complexes. The first-dimension gel lanes of cross-linked hormone–receptor complexes that were solubilized under nonreducing conditions were treated for reduction and electrophoresed on the top of fresh gels.

of SES, the hormone–receptor complexes were not noticeable. As the concentration of the reducing agent increased, these bands were diminished whereas smaller complex bands, the 102 and 74 kDa for ^{125}I-hCG–receptor and the 83 and 65 kDa for ^{125}I-FSH–receptor, appeared. In order to determine the relationship between these bands, gel lanes of cross-linked samples that were solubilized without reducing agents were cut, treated with the reducing agent, and electrophoresed on the top of fresh gels (Figure 7). The results demonstrate that the 102- and 74-kDa complexes were released from the high molecular weight ^{125}I-hCG–receptor complex corresponding to the 136-kDa one. For the cross-linked ^{125}I-FSH–receptor complexes, the 65- and 83-kDa complexes were released from the 117-kDa complex. It is evident that all of the components of the cross-linked hormone–receptor complexes (Table II) were disulfide-linked. The existing evidence cannot, however, prove whether the 18-kDa component of the ^{125}I-hCG–receptor complexes is disulfide-linked with other components. Since the hormones did not form hormone–receptor complexes in the absence of cross-linking and the hormone bands were not released from their respective hormone–receptor complexes under reducing conditions, they were not likely to be disulfide linked to their receptors. The hCG $\alpha\beta$ dimer that appeared in two bands under nonreducing conditions merged into one band upon reduction, both in one-dimensional and two-dimensional analysis (Figures 6 and 7). Apparently, two classes of the hCG $\alpha\beta$ dimer coexist under nonreducing conditions.

Since disulfide could spontaneously form by oxidation of sulfhydryls during cell preparation, hormone incubation, and solubilization of proteins, it was necessary to test whether some of the disulfides were intrinsic or artifacts. The autoradiograms were not significantly different when NEM was present from the time of cell isolation to sample solubilization (Figure 8). In addition, Figure 8 shows that the profiles of cross-linked hormone–receptor complexes were not significantly affected by the presence or absence of various protease inhibitors.

Discussion

The present evidence demonstrates extensive homologies in the structures of the hCG receptor and the FSH receptor. Both receptors are composed of several components, suggesting oligomeric structures. The sizes of the components were similar; the 18-kDa, the 22–24-kDa, and the 34-kDa components being found in both receptors. In contrast, the 28-kDa components was observed only in the hCG receptor. The components in both hormone receptors were disulfide-linked, but there is no evidence of disulfide linkages between the hormones and the receptors.

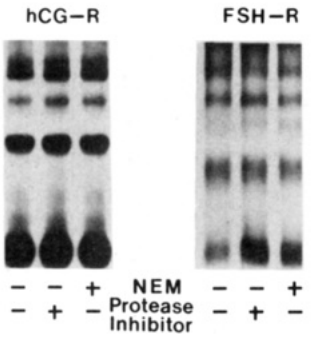

FIGURE 8: Effect of NEM and protease inhibitors on formation of cross-linked hormone–receptor complexes. The sulfhydryl blocking agent 10 mM NEM or various protease inhibitors were present from the cell isolation to electrophoresis. The inhibitors were 1 mM phenylmethanesulfonyl fluoride, 20 μM tosyl-L-lysine chloromethyl ketone, 2 mM EDTA, 100 μM benzamidine, 5 μg/mL leupeptin, 7 μg/mL pepstatin, and 5 mM 6-aminocaproic acid. The last was omitted during cross-linking.

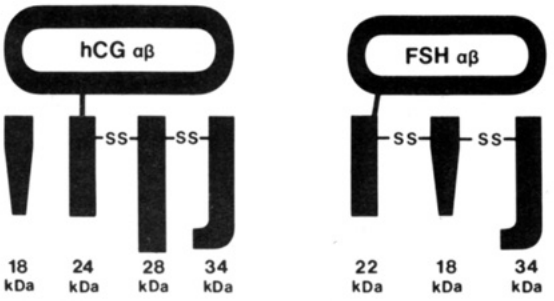

FIGURE 9: Diagrammatic presentation of disulfides between hormone receptor components.

hCG–receptor complexes appear to be preferentially produced by cross-linking of the hCG $\alpha\beta$ dimer to the 24-kDa component, which was disulfide-linked to the 28-kDa component which in turn was disulfide-linked to the 34-kDa component (Figure 9). This conclusion is consistent with the results of the peptide mapping, photoaffinity labeling, affinity cross-linking, and cleaving that demonstrated the 24-kDa component as the preferential site for the hormone cross-linking. The FSH $\alpha\beta$ dimer appears to primarily photoaffinity label or cross-link the 22-kDa receptor component, which was disulfide-linked to the 18-kDa component which in turn was disulfide-linked to the 34-kDa component (Figure 9). Although the apparent sequence of disulfide linkages was not the same, both hormone receptors share components of a similar size, 22–24 kDa, which served as the preferential site for hormone cross-linking.

Disulfides could spontaneously be formed by oxidation of sulfhydryl groups during cell preparation, hormone incubation, and solubilization of proteins. There is also the possibility of new disulfide formation and disulfide exchanges that could be initiated by free sulfhydryl groups (Liu, 1977). These can be, however, quantitatively blocked with NEM at neutral pH (Means & Feenly, 1971). Our results demonstrate that free sulfhydryl groups were not involved in formation of intercomponent disulfides and that the disulfides were intrinsic and present before the cell isolation (Figure 8).

The hormone receptor components identified in this study appear to be subunits of the receptor, since addition of the protease inhibitors 1 mM phenylmethanesulfonyl fluoride, 20 μM tosyl-L-lysine chloromethyl ketone, 5 mM 6-aminocaproic acid, 2 mM EDTA, 100 μM benzamidine, 5 μg/mL leupeptin, and 7 μg/mL pepstatin did not alter the cross-linking and cleaving profile (Figure 8). This combination represents inhibitors for all four major classes of proteases (serine, thiol, metal-dependent, and carboxyl). These inhibitors were included in all experimental buffers except that 6-aminocaproic acid was omitted during cross-linking. The possibility that proteolysis occurs in the intact cells or during cross-linking, washing, and solubilization steps in our procedure even in the presence of added protease inhibitors cannot be rigorously eliminated. However, the incremental photoaffinity labeling, cross-linking, cleaving, and reduction of cross-linked complexes that were reproduced independent of the protease inhibitors and the source of the hormone receptor (the cell surface, the isolated membrane, and Triton X-100 extracts) cannot be simply explained in terms of proteolytic effects. Whether or not the components identified in this study are intrinsic receptor subunits or proteolytic products has yet to be determined by more direct methods. In fact, there was a report of proteolytic degradation of cross-linked hCG–receptor complexes (Kellokumps & Rajaniem, 1985). They have suggested that the smaller hCG–receptor complexes were produced on rat corporal luteal membranes by proteolysis of the largest complex equivalent to the 136-kDa complex in this study and this cleavage was activated by binding of the hormone to the receptor in situ but not after receptor extraction in Triton X-100. Recently, we have purified the hCG receptor on an hCG affinity column after extraction in Triton X-100 and then photoaffinity labeled it with ABG-^{125}I-hCG. The autoradiographic band profile of hCG–receptor complexes was virtually identical with that seen in Figure 1. Obviously, there was not less receptor degradation, were there any, upon binding of the hormone to the solubilized and affinity-purified receptor. We are pursuing this problem further. Our results are similar to those reported for affinity cross-linking of the hCG receptor (Rebois, 1982; Huang & Menon, 1984) and the FSH receptor (Branca et al., 1985). In addition, we have presented molecular weight estimates of receptor components and evidence for intercomponent disulfides and for homologies in the receptor structures. The distinct peptide maps of hCG–receptor complexes and FSH–receptor complexes suggest that the receptor components primarily cross-linked to hCG and to FSH were not entirely the same. It is also possible that the distinct peptide maps were generated by cross-linking identical subunits to the two different hormones or by varying modes of hormone bonding, cross-linking, or both.

LH and FSH are glycoprotein hormones of pituitary origin. These two hormones and hCG, a placental glycoprotein hormone, consist of two noncovalently associated, dissimilar subunits designated α and β. Within the same mammalian species, the α subunits of these hormones have identical amino acid sequences. In contrast, the β subunits have distinct sequences and account for the hormone specificity. Binding and the subsequent biological response are, however, produced primarily by the $\alpha\beta$ dimer (Ward, 1978; Pierce & Parson, 1981; Roche & Ryan, 1985). The similarity in the oligomeric structures of the FSH receptor and the LH receptor suggests an intriguing and local possibility of the parallel evolutionary conservation in the glycoprotein hormones and their receptors.

Registry No. LH, 9002-67-9; FSH, 9002-68-0; hCG, 9002-61-3.

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Characterization and Partial Purification of Cardiac Sarcoplasmic Reticulum Phospholamban Kinase†

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ABSTRACT: Phospholamban, the cardiac sarcoplasmic reticulum proteolipid, is phosphorylated by cAMP-dependent protein kinase, by Ca^{2+}/phospholipid-dependent protein kinase, and by an endogenous Ca^{2+}/calmodulin-dependent protein kinase, the identity of which remains to be defined. The aim of this study was therefore to characterize the latter kinase, called phospholamban kinase. Phospholamban kinase was purified approximately 42-fold with a yield of 11%. The purified fraction exhibits a specific activity of 6.5 nmol of phosphate incorporated into exogenous phospholamban per minute per milligram of protein. Phospholamban kinase appears to be a high molecular weight enzyme and presents a broad substrate specificity, synapsin-1, glycogen synthase, and smooth muscle myosin regulatory light chain being the best substrates. Phospholamban kinase phosphorylates synapsin-1 on a M_r 30 000 peptide. The enzyme exhibits an optimum pH of 8.6, a K_m for ATP of 9 μM, and a requirement for Mg^{2+} ions. These data suggest that phospholamban kinase might be an isoenzyme of the multifunctional Ca^{2+}/calmodulin-dependent protein kinase. Consequently we have searched for M_r 50 000–60 000 phosphorylatable subunits among cardiac sarcoplasmic reticulum proteins. A M_r 56 000 protein was found to be phosphorylated in the presence of Ca^{2+}/calmodulin. Such phosphorylation alters the electrophoretic migration velocity of the protein. In addition, this protein that binds calmodulin was always found to be present in fractions containing phospholamban kinase activity. This M_r 56 000 protein is therefore a good candidate for being a subunit of phospholamban kinase. However, the M_r 56 000 calmodulin-binding protein and the M_r 53 000 intrinsic glycoprotein which binds ATP are two distinct entities.

Muscle relaxation occurs when Ca^{2+} is removed from myofibrils by active transport across the sarcoplasmic reticulum (SR)[1] membrane through a membrane bound (Ca^{2+},Mg^{2+})-ATPase. In cardiac muscle the rate of relaxation is dependent on both the adrenergic status (Wray et al., 1973; Kirchberger et al., 1974) and the cytosolic free Ca^{2+} concentration (Lopaschuk et al., 1980; Plank et al., 1983). These stimulations of Ca^{2+}-dependent ATPase and Ca^{2+} uptake are associated with phosphorylations of phospholamban, a membrane-bound M_r 24 000 proteolipid.

The involvement of these multiple pathways in vivo is under investigation. Although the action of β-adrenergic agonists in systole abbreviation is widely accepted to proceed through phosphorylation of phospholamban (Le Peuch et al., 1980; Kranias & Solaro, 1982), there is no consensus of opinion concerning the importance of phospholamban Ca^{2+}/calmodulin-dependent phosphorylation. Le Peuch et al. (1980) have shown that fluphenazine induces an inhibition of phospholamban phosphorylation in intact rat myocardium. Lindemann and Watanabe (1985) have reported, however, that direct physiological increases in cytosolic free Ca^{2+} do not stimulate phospholamban phosphorylation in vivo. These authors suggest that the Ca^{2+}/calmodulin-dependent phosphorylation of phospholamban requires the presence of both cAMP and free Ca^{2+}. Characterization and identification of phospholamban

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[1] Abbreviations: SR, sarcoplasmic reticulum; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; PEG, poly(ethylene glycol); SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; Hepes, 4-(2-hydroxymethyl)-1-piperazineethanesulfonic acid; Endo H, endo-β-*N*-acetylglucosaminidase H; 5′-FSO_2BzAdo, 5′-[*p*-(fluorosulfonyl)-benzoyl]adenosine; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Me_2SO, dimethyl sulfoxide; ATPase, adenosinetriphosphatase; TCA, trichloroacetic acid.

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