Endocrine (1995) 3, 907-911

Receptor binding dependent structural changes in human choriogonadotropin: photochemical inter-subunit crosslinking

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Activation of surface receptors is thought to occur in multiple transient steps with conformational adjustments of hormones and receptors beginning from the initial hormone-receptor contact. In this study, we have established a sensitive photochemical crosslinking method to detect structural change of hCG upon receptor binding. hCG consists of an α subunit and a β subunit. Free α subunit was derivatized with photosensitive reagents and reassociated with unmodified β subunit. Reassociated hCG αβ dimer was capable of high affinity receptor binding and activation. The reagents attached to the α subunit were capable of crosslinking the α subunit to the β subunit. However, the extent of inter-subunit cross-linking in solution was two - three fold greater than inter-subunit crosslinking after hCG bound to the receptor. This difference indicates a novel structural change at the subunit interface in response to hCG binding to the receptor. Although highly unlikely, other microenvironmental factors might have interfered with the crosslinking efficiency without impacting the structure of hCG. This study lays the ground work to precisely define the location and nature of the change. Such information will be crucial for the understanding of the molecular mechanism of the hormone-receptor interaction and receptor activation.

Keywords: hCG, subunit; crosslinking; receptor; binding

Introduction

Hormonal action depends upon receptor activation. In spite of this crucial role, the mechanism of receptor activation is poorly understood. Surface receptors are activated by ligand binding. However, not all hormone binding results in receptor activation to generate signals, an indication of other requirements. Existing data suggest that the conformation of hormones and receptors must correctly change and adjust during multiple transient steps from the initial hormone-receptor contact to receptor activation (Ji *et al.*, 1995). The evidence for such crucial conformational adjustments have been elusive except for growth hormone and its receptor (de Vos *et al.*, 1992). The conformational adjustment of the growth hormone-receptor complex was elegantly demonstrated by the crystal structure of the complex. However, crystal structures of most hormone-receptor complexes are difficult to obtain and there is a need for other less difficult technologies to determine and to study conformational adjustments of numerous hormones and receptors during their complementary interactions and receptor activation.

The goal of this study is to detect conformational changes of hCG upon receptor binding. This glycoprotein hormone was used, taking advantage of its subunit structure to analyse whether the subunit interaction underwent changes during receptor binding and activation. hCG is composed of two noncovalently associated glycoprotein subunits, the ~15 kD α subunit and the ~20 kD β subunit. Other members of the glycoprotein hormone family, LH, FSH and TSH, also have the two subunits. The α subunits are encoded by a single gene and have an identical amino acid sequence (Boothby *et al.*, 1981). The β subunits differ for each hormone since they are encoded by distinct genes (Gharib *et al.*, 1990; Jameson & Hollenberg, 1993). Only the αβ dimer is capable of high affinity receptor binding to induce biological responses and the dissociated subunits lose high affinity binding activity (Roche & Ryan, 1985).

The LH/CG receptor has high and low affinity contact sites for hCG and the low affinity site alone activates the receptor. The receptors for the glycoprotein hormones comprise a subfamily of the seven transmembrane receptor super family (Probst *et al.*, 1992). These receptors have an N-terminal extracellular extension, seven transmembrane domains, three cytoplasmic loops and three extracellular loops that link the seven transmembrane domains, and a cytoplasmic tail. The N-terminal extensions (halves) of the glycoprotein hormone receptors are the largest with ~350 amino acids, in comparison to the 11-30 amino acids long N-terminal extensions of adrenergic receptors (Probst *et al.*, 1992). These N-terminal halves of the glycoprotein hormones are responsible for high affinity hormone binding (Tsai-Morris *et al.*, 1990; Xie *et al.*, 1990; Ji & Ji, 1991a; Seetharamaiah *et al.*, 1994; Davis *et al.*, 1995) but they are incapable of inducing hormone action (Ji & Ji, 1991b; Remy, *et al.*, 1993).

Recent mutational analyses of the LH/CG receptor indicate the spatial and temporal distinction of hormone binding and receptor activation (Ji *et al.*, 1993; Ji *et al.*, 1995). Activation of the LH/CG receptor involves low affinity binding (Ji & Ji, 1991b; Yoo *et al.*, 1993) at the membrane associated C-terminal half of the receptor (Ji & Ji, 1993; Ji *et al.*, 1993; Remy, 1993). This low affinity binding occurs after the initial high affinity contact of hCG with the receptor (Ji *et al.*, 1993). These are in agreement with multiple step hCG-receptor interactions (Moyle, 1980; Roche & Ryan, 1985) and structural adjustments of hormones and receptors (Ji *et al.*, 1993; Ji *et al.*, 1995). The FSH receptor also behaves similarly. Kinetic studies of the FSH receptor suggest high and low affinity hormone bindings (van Loenen *et al.*, 1994a; van Loenen *et al.*, 1994b). The low affinity binding alone is responsible for receptor activation.

Results

The N-hydroxysuccinimide ester (NHS) of 4-azidobenzoic acid (ABG) was used to derivatize the hCG α subunit with or without radioiodination and the derivatized α subunit was recombined with untreated hCGβ. Reconstituted ABG-α/β is capable of receptor binding and inducing cAMP synthesis with affinities and efficacy slightly less than those of intact hCG (Figure 1). These results indicate that the derivatization of the α subunit and reassociation with the β subunit did not significantly impair the activity of the reconstituted hormones. This is consistent with past reports that chemically crosslinked glycoprotein hormones retain full or partial biological activity (Combarnous & Hennen, 1974; Burleigh *et al.*, 1978; Mori *et al.*, 1978; Parson & Pierce, 1979; Weare &

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Received 28 July 1995; accepted 26 September 1995

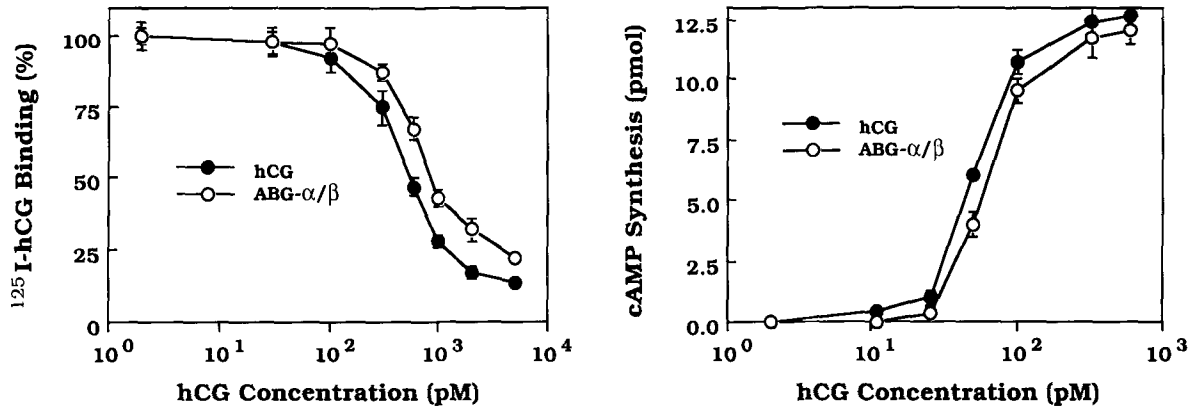

Figure 1 Receptor binding and cAMP induction: (Left Panel). Porcine granulosa cells were incubated with a constant amount of ^{125}I-hCG (4×10^8 CPM/μg) in the presence of increasing concentrations of hCG or ABG-α/β. After washing cells, radioactivity associated with cells were counted. Experiments were repeated twice, each time in duplicate. The results were analysed by Student's *t*-test. The Kd values obtained from Scatchard plots are 523±42 pM ($P<0.05$) and 681±57 pM ($P<0.05$), respectively for hCG and ABG-α/β. (Right Panel). A murine Leydig tumor cell line was incubated with increasing concentrations of hCG or ABG-α/β and intracellular cAMP concentrations determined. The EC_{50} values for cAMP induction are 51.3±4.3 pM ($P<0.05$) and 65.4±5.7 pM ($P<0.05$), respectively for hCG and ABG-α/β

Reichert Jr, 1979a; Weare & Reichert Jr, 1979b; Gordon & Ward, 1985; van Dijk & Ward, 1993). The divergent biological activities of modified and reconstituted hormones are likely due to variations to intermolecular cross-linking. (van Dijk & Ward, 1993), the extent of modifications, and the chemical and physical properties of the modifying groups (Gordon & Ward, 1985). Therefore, it is important to control the extent of derivatization and the chemical and physical properties of reagents.

Figure 2 shows photoaffinity crosslinking of ABG-^{125}I-α/β. When ABG-^{125}I-α/β bound to the LH/CG receptor on granulosa cells was irradiated with 5 Xenon flashes, the αβ dimer band appeared (Figure 2A2). The same sample without UV irradiation show only the α subunit band (Figure 2A1). The β subunit is not radioactively labeled and therefore, does not appear on the autoradiograph although it is located in the middle of the α and the αβ dimer bands (Ji & Ji, 1981). Under the condition of reduction, denaturation and solubilization, uncross-linked ABG-^{125}I-α/β dimer is completely dissociated into the individual subunits (Figure 2A1-3). These results indicate that inter-subunit crosslinking of the αβ dimer requires photolysis and only the α subunit is radioactively labeled. When the sample was irradiated with 15 flashes, two bands crosslinked hormone/receptor complexes appeared in addition to the inter-subunit crosslinked αβ dimer. The two bands are likely to represent crosslinked ABG-^{125}I-α/receptor and ABG-^{125}I-α/β/receptor complexes, respectively (Figure 2A3), because they were not produced when free ABG-^{125}I-α/β in solution was flashed 15 times. Taken together, these results indicate that inter-subunit crosslinking of the αβ dimer occurred before hormone-receptor complexes were cross-linked. Further, a condition is established in which crosslinking occurs primarily between the α and β subunits, but not between the hormone and receptor. Under this condition, the radioactivity of inter-subunit crosslinked αβ dimer accounted for 9% of the total ABG-^{125}I-α radioactivity in the gel lane (Figure 2A2). In the following studies, this condition for inter-subunit was used.

In Figure 2B, after granulosa cells were incubated with ABG-^{125}I-α/β at 4°C, the incubation mixture was irradiated with 5 flashes and centrifuged. Cell pellets were washed twice. The incubation supernatant (Figure 2B2), wash media (Figure 2B3) and cell pellet (Figure 2B4) show the cross-linked αβ dimer band. Controls were ABG-^{125}I-α/β in fresh media which was irradiated with 5 flashes (Figure 2B1) and cells complexed with ABG-^{125}I-α/β which were not trated with UV flashes (Figure 2B5). The radioactivity of inter-subunit

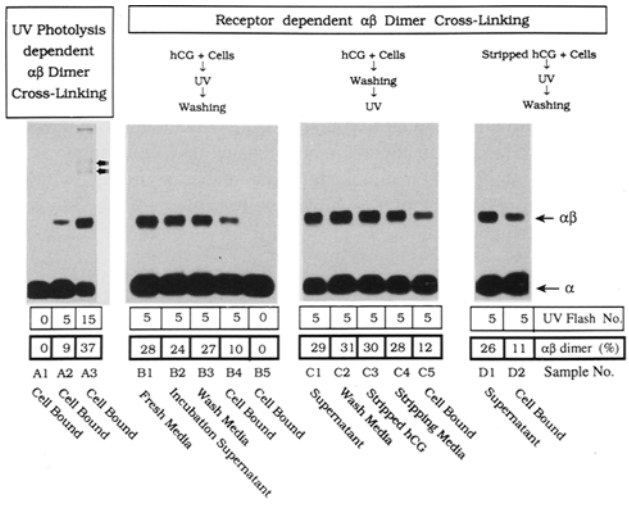

Figure 2 Autoradiographs of photoaffinity crosslinked hCGαβ dimer: hCGαβ dimers were crosslinked four different ways (A–D). (**A**) Granulosa cells were incubated with ABG-^{125}I-α/β, irradiated with 0, 5 or 15 Xenon flashes and washed. After solubilization the samples were electrophoresed and the resulting gel was dried and exposed to X-ray film for autoradiograph. Details are described in Materials and methods. The positions of radioactive α and αβ bands are indicated. The non-radioactive hCGβ band cannot be seen and is present in the middle between the α and αβ bands (Ji & Ji, 1981). Two arrows mark crosslinked hormone-receptor complexes. (**B**). Cells were incubated with ABG-^{125}I-α/β, irradiated with 5 Xenon flashes and washed. Electrophoresed samples are fresh ABG-^{125}I-α/β in fresh media (B1), the incubation supernatant (B2), the first wash media (B3) and cells complexed with ABG-^{125}I-α/β (B4). As a negative control, cells complexed with ABG-^{125}I-α/β were electrophoresed without UV irradiation (B5). (**C**) Cells were incubated with ABG-^{125}I-α/β and washed. The incubation supernatant (C1) and the first wash media (C2) were photolyzed and electrophoresed. Part of cells complexed with ABG-^{125}I-α/β irradiated and electrophoresed (C5). ABG-^{125}I-α/β bound to part of cells were removed and stripped ABG-^{125}I-α/β was irradiated (C3). Fresh ABG-^{125}I-α/β in stripping media (C4). Cells complexed with ABG-^{125}I-α/β (C5). (**D**) Cells were incubated with stripped ABG-^{125}I-α/β and photolyzed and washed. The incubation media (D1) and cells complexed with stripped ABG-^{125}I-α/β were electrophoresed. Crosslinked hCG αβ dimer appeared in Figure 2 were analysed by Student's *t*-test. It was 2.8±2 fold higher ($P<0.01$) when hCG was in solution than hCG was bound to the receptor. Experiments were individually repeated 5–20 times and the results were consistent with the data in this figure

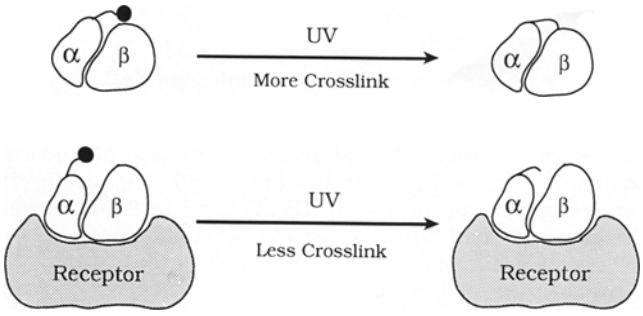

Figure 3 Hypothetical presentation of receptor dependent inter-subunit crosslinking of hCG. The intersubunit interaction of hCGα and β changes upon receptor binding. As a result the inter-subunit crosslinking by attached photosensitive reagent of the hCG dimer in solution (upper drawing) is more efficient than inter-subunit crosslinking of the hCG dimer bound to the receptor (lower drawing). The change in the inter-subunit interaction is exaggerated

crosslinked αβ dimer was 10% of the total radioactivity of the hormone for receptor bound ABG-^{125}I-α/β (Figure 2B5). On the other hand, it was 24–28% for free ABG-^{125}I-α/β in solution (Figure 2B1-4). This less efficient inter-subunit crosslinking of receptor-bound ABG-^{125}I-α/β indicates structural change in the vicinity of the reagent upon hormone binding to the receptor (Figure 3). Alternatively, the hormone preparation might be comprised of two groups of different structures, a structure more favorable for inter-subunit crosslinking and the other less favorable. If the less favorable population had a higher receptor binding affinity than did the more favorable population, inter-subunitcross-linking of receptor-bound ABG-^{125}I-α/β would be less than that of the more favorable population.

To test these possibilities, ABG-^{125}I-α/β bound to cells were separated without photolysis from unbound ABG-^{125}I-α/β. They were independently photolyzed and electrophoresed (Figure 2C1 and 2C2). In addition, ABG-^{125}I-α/β bound to cells were removed and stripped ABG-^{125}I-α/β was photolyzed (Figure 2C3). Twenty eight percent of stripped ABG-^{125}I-α/β was crosslinked (Figure 2C3). These results indicate that ABG-^{125}I-α/β bound to cells is not different from free ABG-^{125}I-α/β. To further verify this conclusion, stripped ABG-^{125}I-α/β was re-introduced to fresh granulosa cells and photolyzed. Free ABG-^{125}I-α/β was removed from cell bound ABG-^{125}I-α/β. Inter-subunit crosslinks were 11% of the total for hormone bound to cells (Figure 2D2) and 26% for free hormone. (Figure 2D1). These results clearly indicate that stripped ABG-^{125}I-α/β behaved the same as the general population of free ABG-^{125}I-α/β. An excess of untreated hCG blocked binding of ABG-^{125}I-α/β and stripped ABG-^{125}I-α/β (data not shown), an indication of their binding specificity. Taken together, these results indicate that the structure of bound ABG-^{125}I-α/β is different from that of unbound ABG-^{125}I-α/β. Further, the structural change is reversible.

Discussion

Our results demonstrate that ABG coupled to the α subunit is capable of covalently cross-linking the β subunit. This inter-subunit crosslink occurs prior to crosslinking of the hormone to the receptor. Therefore, inter-subunit crosslink is likely to be independent of cross-linking of the hormone to the receptor and occurs at the αβ subunit interface. In fact, there is evidence that αLys^{49} of LH (equivalent to αLys^{45} of hCG) can be crosslinked to the β subunit (Weare & Reichert Jr, 1979a; Weare & Reichert Jr, 1979b; van Dijk & Ward, 1993) at the αβ interface (Lapthorn *et al.*, 1994; Wu *et al.*, 1994).

ABG-^{125}I-α/β is crosslinked to form the αβ dimer in 2.8 ± 2 fold ($P<0.01$) higher levels in solution than bound to the receptor on cells. This result indicates structural differences in the vicinity of ABG coupled to the hormone derivative present in solution and bound to cells. These structural differences are most likely caused by alterations in the hormone structure when the hormone binds to the receptor (Figure 3). This is not surprising because proteins are known to undergo local and/or global conformational changes when they interact with other proteins, subunits and ligands (Branden & Tooze, 1991). Other microenvironmental factors might have interfered with the crosslinking efficiency. However, it is difficult to conceive some mircoenvironmental factors including steric hiderance, hydrophobicity and electrostatic interference, which could interfere crosslinking reaction without causing minute local changes of the hCG structure.

There are seven potential amino groups for derivatization in the α subunit, the N-terminus, Lys^{44}, Lys^{45}, Lys^{51}, Lys^{63}, Lys^{75} and Lys^{91}. These sites are accessible to chemical modification by some but not all reagents, dependent on the reactivity, concentration and reaction condition of reagents (Gordon & Ward, 1985; Yadav *et al.*, 1994). The N-terminus of the α subunit is present near the N-terminal region of the β subunit. Therefore, both N-terminal regions could be crosslinked. However, they are at the opposite side of the putative receptor interface. If the extent of this particular crosslinking was reduced after receptor binding, it would suggest that receptor binding caused a global change in the hormone structure. Such a change would significantly impact the subsequent hormone-receptor interactions and receptor activation. αLys^{44} and αLys^{45} are near the αβ dimer interface (Bielinska & Boime, 1992; Lapthorn *et al.*, 1994; Wu *et al.*, 1994). ABG attached to them are capable of reaching and crosslinking the β chain through a number of reactive groups as described above. Further, they are not too far from the putative receptor interface. Therefore, it is possible for them to undergo local structural changes upon receptor binding without a global change. αLys^{63} is near the β^{93-100} putative receptor binding determinant (Ward & Moore, 1979; Keutmann *et al.*, 1989; Lapthorn *et al.*, 1994; Wu *et al.*, 1994) although not at the putative receptor interface. Therefore, ABG coupled to it could reach and crosslink several reactive groups of βArg^{94}, Arg^{95}, Ser^{96}, βThr^{97}, βThr^{98}, βAsp^{99}. These could be impacted by receptor binding. αLys^{51}, αLys^{75} and αLys^{91} project toward the putative receptor interface (Lapthorn *et al.*, 1994; Wu *et al.*, 1994) and reagents attached to them are expected to reach and crosslink the receptor. αLys^{91} interacts with Asp^{397} of the receptor during or after the transition from the high affinity binding to low affinity binding (Ji *et al.*, 1993). It cannot be, however, ruled out that 10Å ABG coupled to these Lys residues crosslinks to the β subunit.

Structural changes of the hormone upon receptor binding are likely to be crucial for receptor activation (Ji *et al.*, 1995). There are high and low affinity hormone-contact sites in the receptor. The high affinity site is in the extracellular N-terminal half of the receptor (Tsai-Morris *et al.*, 1990; Xie *et al.*, 1990; Ji & Ji, 1991a) and the low affinity site is in the membrane associated C-terminal half (Ji & Ji, 1991a; Ji & Ji, 1991b; Remy, 1993). The low affinity site alone is capable of activating the receptor (Ji & Ji, 1991b; Remy, 1993). Mutational analyses indicate that hCG initially binds to the high affinity site and subsequently to the low affinity site. Kinetic studies on the interaction of hCG and its receptor also suggests multiple steps (Moyle, 1980; Roche & Ryan, 1985). It has been suggested that the hormone undergoes structural changes during the transitions (Ji *et al.*, 1993). This is consistent with the conventional wisdom that protein-protein interactions result in changes of local and/or global structures (Branden & Tooze, 1991). It is worthy to note that these structural changes are reversible according to the results in Figure 2. The reversibility indicates that the structural changes and the hormone-receptor interaction are inter-

dependent. It also shows that the structural changes involve electrostatic, hydrogen and/or van der Waals interactions but not covalent bonds including disulfides. The numerous neighboring disulfides of the cystine knots of hCG, therefore, do not undergo intersubunit disulfide exchange. This is consistent with the electrophoretic separation of uncross-linked α and β subunits in Figure 2.

Lessons learned from this study along with the determination of the precise location of structural changes will be crucial for the understanding of the receptor activation mechanism. In addition, we have shown that photochemical cross-linking will be useful to detect conformational changes of hormone and to determine the sites in other protein and polypeptide hormones. Further, this study opens new opportunities to investigate the structure-function relationship of glycoprotein hormones and their mutants in receptor activation. For examples, there is a number of mutant hCGs and FSHs which are normal in receptor binding but defective in receptor activation (Matzuk *et al.*, 1989; Yoo *et al.*, 1991; Yoo *et al.*, 1993; Zeng *et al.*, 1995). Inter-subunit cross-linking of these defective hormones in solution and bound to their receptors could shed light on the structural adjustments of the hormones during receptor activation and therefore, the mechanisms of receptor activation.

Materials and methods

Radio-iodination, derivatization and reconstitution of hCGs (ABG-α/β and ABG-^{125}I-α/β)

hCG-hCG, hCGα and hCGβ were supplied by National Hormone and Pituitary Program. The N-hydroxysuccinimide ester of 4-azidobenzoic acid (NHS-ABG) was prepared as previously described (Ji *et al.*, 1980). The α subunit (5 μg) was dissolved in 40 μl of 0.1 M sodium phosphate, pH 7.5, and mixed with 10 μl of 0.5 mM NHS-ABG in 0.1 M sodium phosphate, ph 7.5. After incubation for 15 min, 1 mCi ^{125}I-sodium iodide in 10 μl of 0.1 M sodium hydroxide and 7 μl of chloramine-T solution (0.3 mg in 1 ml of water) were added sequentially. They were mixed thoroughly and incubated for 20 s at 25°C. Then 7 μl of sodium metabisulfite (0.66 mg of sodium metabisulfite/ml of PBS) was added, followed by 60 μl of 16% sucrose in PBS. The α subunit was charged to Sephadex G-50 superfine column (0.6 cm × 20 cm) and eluted with 0.1 M sodium phosphate, pH 7.5, and 0.1% gelatin. The α subunit peak was incubated with untreated hCGβ in 0.1 M sodium phosphate, pH 7.5, at 37°C for 16 h according to the reported procedure (Canfield *et al.*, 1971). Reconstituted ABG-^{125}I-α/β was purified on FPLC Superose 12. The yield was ~76% according to the recovered radioactivity. For ABG-α/β, the entire radio-iodination step was omitted. Receptor binding and cAMP assay (Figure 1) were carried out as previously reported (Ji & Ji, 1980; Ji & Ji, 1993).

Photoaffinity crosslinking

Freshly prepared porcine granulosa cells were incubated with ABG-^{125}I-α/β for 10 h at 4°C (to minimize internalization of bound hormones) (Aharonov *et al.*, 1978; Goldstein *et al.*, 1985; Seagaloff & Ascoli, 1981; Yadav *et al.*, 1994) and the incubation mixture was transferred to an ice cold quartz container (2 cm × 4 cm × 1 cm/width, length and depth. It was placed on top of an ACME-LITE model 228A Xenon flash and covered with another ACME-LITE model 228A Xenon flash (Kiehm & Ji, 1977). The flash units were fired simultaneously and with increasing frequency. Each flash lasts ~0.2 ms and minimizes random collisional crosslinks (Kiehm & Ji, 1977). Treated cells were centrifuged at 1,000 X g for 10 min and cell pellets were rinsed twice. The washed cells were solubilized in 3% sodium dodecylsulfate and 100 mM dithiothreitol. Equal amounts of radioactivity were electrophoresed on polyacrylamide gels (Figure 2A). Gels were dried on filter paper and exposed to X-ray film. Radioactive bands on dried gels were cut and the radioactivity was counted.

In the second approach, the incubation mixture was irradiated with five flashes and centrifuged. After saving the incubation supernatant, cells were washed twice and the first wash media were saved. A third wash was not necessary since no significant radioactivity was removed from the cells. The washed cell pellet, the incubation supernatant and the first wash media were electrophoresed (Figure 2B). In the third approach, cells were incubated with ABG-^{125}I-α/β for 10 h at 4°C and centrifuged. Cell pellets were washed twice to remove unbound ABG-^{125}I-α/β. To remove and to recover specifically bound ABG-^{125}I-α/β from cells, washed cells were incubated in ice-cold 50 mM glycine-HCl, 100 mM NaCl (pH 3.0) for 2 min at 4°C and rinsed once more with the same buffer. The acid solutions were combined and stripped ABG-^{125}I-α/β concentrated (Figure 2C3). This procedure has been used to remove and study surface bound hormones (Aharonov *et al.*, 1978; Goldstein *et al.*, 1985) and verified surface bound hCG (Seagaloff & Ascoli, 1981; Yadav *et al.*, 1994). In the fourth approach, freshly prepared granulosa cells were incubated with stripped ABG-^{125}I-α/β for 10 h at 4°C and the incubation mixture was irradiated with five flashes. After centrifugation, the incubation supernatant was saved and cells were washed twice. The incubation supernatant and cell pellet were solubilized and electrophoresed as before (Figure 2D).

Acknowledgements

This work was supported by Grant HD 18702 from the National Institutes of Health.

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