Pergamon

J. Steroid Biochem. Molec Biol Vol 48, No. 1, pp. 139–144, 1994

0960-0760/94 $6 00 + 0 00

0960-0760(93)E0003-P

Identification of the Cysteine in the Steroid-binding Site of Human Corticosteroid Binding Globulin by Site-directed Mutagenesis and Site-specific Chemical Modification

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Binding of corticosteroids by human corticosteroid binding globulin (hCBG) is thought to involve interaction with one of its two cysteine residues (Cys_{60} and Cys_{228}). To identify which of the two cysteine residues mediates steroid binding, we have produced mutant hCBGs containing serine or alanine in place of Cys_{228} by site-directed mutagenesis. Alteration of Cys_{228} to serine or alanine does not change the steroid binding affinity of hCBG, demonstrating that Cys_{228} is not involved in the binding interaction. This finding strongly suggests that Cys_{60} is the functionally important cysteine. By modifying the wild-type and mutant hCBGs with the sulfhydryl-specific reagents *N*-ethylmaleimide, iodoacetamide, and sodium tetrathionate, we have demonstrated that Cys_{60} is present at the steroid binding site, and that it may be directly involved in steroid binding. This result also identifies Cys_{60} as the accessible cysteine reported in previous studies.

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INTRODUCTION

Human corticosteroid binding globulin (hCBG) is a serum glycoprotein that binds cortisol and progesterone with high affinity. Apart from maintaining a balance of bound and free steroids in the circulation, hCBG may be involved in release of cortisol at the site of inflammation, following cleavage of hCBG by neutrophil-derived elastase [1].

We set out to investigate the structure–function relationships within CBG, and to understand the basis for steroid specificity. Affinity labeling studies have suggested that one cysteine residue is present within the steroid binding site of hCBG [2]. The mature polypeptide chain of hCBG contains two cysteine residues, Cys_{60} and Cys_{228} [3], and these residues do not form an intramolecular disulfide bond [2]. It is not yet resolved whether the free sulfhydryl (SH) group of the residue associated with the steroid binding site is involved in the steroid–protein interaction or in maintaining the structural integrity of the steroid binding site. Comparison of the cDNA sequences of human, rat, and rabbit CBG revealed that Cys_{228} is conserved among these species [3–5]. Moreover this residue is present within a conserved peptide sequence of 40 amino acids [6]. This observation offers a preliminary indication that Cys_{228} is the residue present in the steroid binding site.

To identify which of the two cysteine residues is associated with the steroid binding site of hCBG, we created two mutant hCBGs, in which the Cys_{228} is replaced by serine or alanine (designated as hCBG-Ser_{228} and hCBG-Ala_{228}, respectively). Production of these two mutant proteins served several purposes. Observation of a change in the steroid binding properties of these mutant proteins would provide strong evidence that Cys_{228} is involved in steroid binding. If, however, the mutant proteins containing only Cys_{60} are capable of binding steroid, these recombinant proteins would comprise excellent models to address the role of this residue using chemical modification with SH-specific reagents.

Here we report expression of mutant hCBGs in insect cells, using the baculovirus system in which we have previously shown high levels of expression

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Received 15 July 1993, accepted 5 Oct 1993

of bioactive recombinant hCBG [7]. Contrary to our expectation, comparison of the steroid binding properties and steroid specificities of the wild-type and mutant hCBGs showed that Cys_{228} is not essential for steroid binding. Using SH-specific reagents with the single cysteine-containing mutant proteins, we instead have identified Cys_{60} as being present in the steroid binding site.

MATERIALS AND METHODS

Site-directed mutagenesis

Oligonucleotide directed mutagenesis was carried out in a two step polymerase chain reaction (PCR) as described by Landt *et al.* [8] and as outlined in Fig. 1. Mutagenic oligonucleotides were designed to change Cys_{228} to serine or alanine. Two synthetic oligonucleotides were synthesized, corresponding to bp 771–797 of the cDNA in the antisense direction:

Oligo 3: 5′ CTG CAC CAG CTG GCT GGG GAG CTC TGA 3′
Oligo 4: 5′ CTG CAC CAG CTG GGC GGG GAG CTC TGA 3′

Oligos 3 and 4 contain a codon for serine and alanine in place of cysteine, respectively. The base changes within these codons are underlined.

The 5′ universal primer (Oligo A) was: 5′ GCC AGC AGA CAG ATC AAC 3′ (bp 531–548 of the sense strand of the cDNA). The 3′ universal primer (Oligo B) was: 5′ TCG TGT CCC GGC TCA GTG 3′ (bp 871–888 of the cDNA in the antisense direction). The 5′ and 3′ universal primers flank the Sal 1 and Bsp E1 restriction sites, respectively, enabling the final PCR fragments to be cloned into hCBG cDNA at those sites. PCR was carried out in a DNA thermal cycler (Perkin Elmer Cetus).

A first round of PCR was performed in 100 μl 1 × Taq polymerase buffer containing 2 μM oligo A, 2 μM mutagenic oligonucleotide (oligo 3 or 4), 2 ng of pGEM1-CBG DNA [7], 50 μM dNTP mixture, and 10 U of Taq DNA polymerase (Promega). 30 cycles of amplification were carried out, each consisting of 60s at 94°C, 60s at 55°C for oligo 3 or 58°C for oligo 4, and 45s at 72°C. The resulting fragments were gel purified [9]. The second PCR reaction was carried out as described for the first PCR, but in this case the entire intermediate fragment served as the 5′ primer and oligo B was used as a 3′ primer, the annealing temperature was also changed to 45°C. The purified PCR products of this reaction were digested with Sal 1 and Bsp E1, and the products were gel purified and cloned into pGEM1-CBG which has been cleaved with Sal 1 and Bsp E1. The cloning protocol was as described previously [9]. The presence of the desired mutations was confirmed by sequencing both strands of the mutagenized insert (Sal 1 to Bsp E1). The complete hCBG cDNA sequences were then cloned in the baculovirus derived vector pVL1393 to generate pVL1393CBG–Ser_{228} and pVL1393CBG–Ala_{228}; these were then used to produce recombinant baculovirus as described previously [10, 7].

Cell culture

Spodoptera frugiperda (*Sf 9*) cells were maintained in Grace's insect cell culture medium supplemented with TC yeastolate, lactalbumin hydrolysate (each at 3.33 gm/l, Difco) and 10% fetal bovine serum (Gibco). This medium is known as TNMFH–10% FBS. For serum-free medium culture, Grace's medium containing TC yeastolate and lactalbumin hydrolysate was supplemented with 1% ITS supplement containing insulin, transferrin, selenius acid, and bovine serum albumin (BSA) (Collaborative Research). Alternatively, sf900 medium (Gibco BRL) was used. *Sf 9* cells were always maintained at 27°C.

Infection and metabolic labeling

To synthesize wild-type and mutant hCBGs *in vivo*, *Sf 9* cells were infected with the corresponding recombinant viruses at a multiplicity of infection of 10–25 infectious units per cell. Radiolabeling, immunoprecipitation, and SDS–PAGE were performed as described previously [7].

Steroid binding

A binding assay employing [^{3}H]cortisol (40–50 Ci/mmol, New England Nuclear) was carried out as described previously [11]. Prior to assay, cell lysate samples were diluted with 10 mM Tris–HCl (pH 8.0), and medium samples were concentrated and dialyzed against the same buffer.

Chemical modification with SH-specific reagents

Sf 9 cell-derived medium containing wild-type or mutant hCBGs was concentrated several fold and dialyzed against 10 mM Tris–HCl (pH 8.0), using Centricon filter apparatus (Amicon). The final samples contained nanomolar concentrations of hCBG. 320 μl of each sample were treated with 50 mM dithiothreitol (DTT) (final concentration) for 1 h at room temperature. The reduced samples were then treated with 200 mM *N*-ethylmaleimide [12], 100 mm iodoacetamide [13] or 200 mm sodium tetrathionate [14] at room temperature. Excess reagents were removed by passing the samples through a spin column of Sephadex G25 equilibrated in 10 mM Tris–HCl, pH 7.4. Finally, [^{3}H]cortisol binding was measured in the eluate fraction.

RESULTS

In vivo expression of mutant hCBG

To investigate the role of cysteine residues in steroid binding by hCBG, the codon for Cys_{228} in hCBG cDNA was changed by site-directed mutagenesis to encode serine or alanine. The resulting hCBG–Ser_{228} and hCBG–Ala_{228} sequences were then expressed in *Sf 9* insect cells, using recombinant baculoviruses as vectors. To monitor the expression of mutant hCBGs

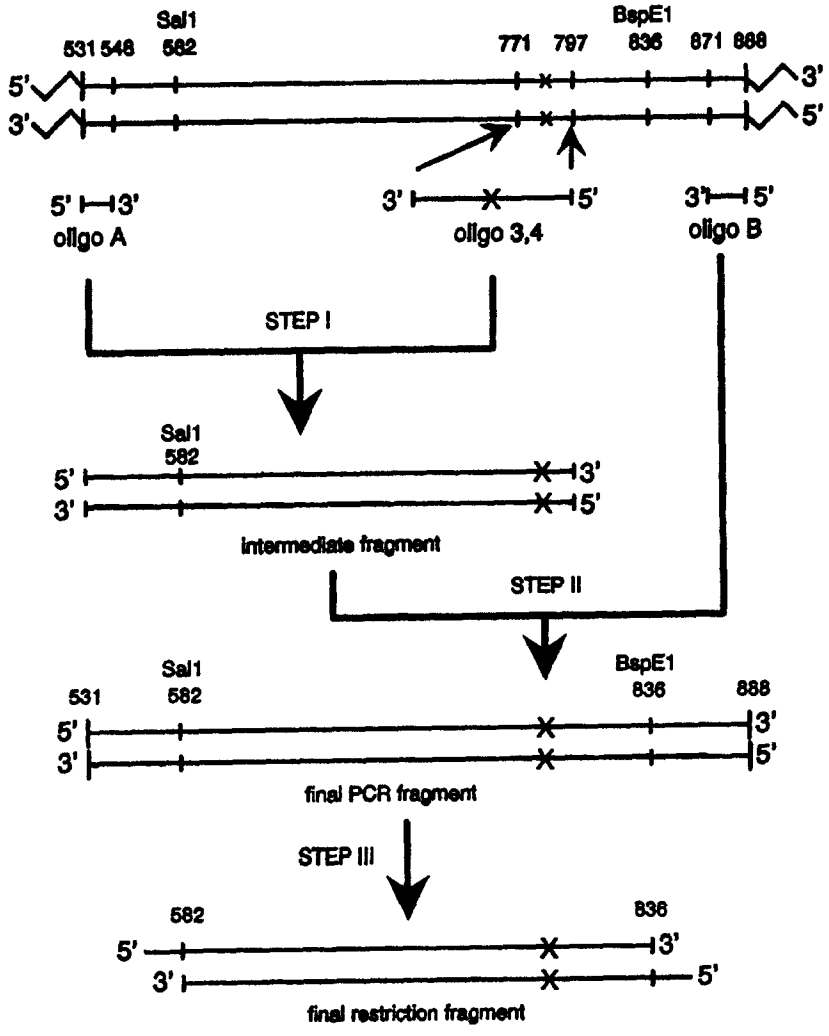

Fig. 1. Strategy for site-directed mutagenesis by PCR. Step 1: the mutagenic primer (oligo 3 or 4) and the 5′ primer (oligo A) are used in the first PCR to generate the intermediate fragment as indicated. Step 2: in the second PCR, the entire intermediate fragment is used as the 5′ primer along with the 3′ primer (oligo B) to yield the final PCR fragment. Step 3: the final PCR fragment was digested with Sal I and BspE I to obtain the fragment used for cloning.

and the kinetics of hCBG secretion, a pulse-chase labeling experiment was performed. The infected cells were metabolically labeled with [^{35}S]methionine. Intra- and extracellular hCBG were isolated by immunoprecipitation with antihCBG antibody and were resolved by SDS–PAGE [Fig. 2(a)]. Both mutant hCBGs were expressed as efficiently as wild-type hCBG. These mutant proteins exhibit an identical distribution of molecular forms, indicating that they undergo similar posttranslational modifications as does wild-type hCBG. Furthermore, the kinetics of secretion of these mutant proteins are similar to those of wild-type hCBG [Fig. 2(b)]. After 2 h of chase, ~40% of pulse-labeled hCBG–Ser_{228} and ~50% of hCBG–Ala_{228} are secreted.

Biological activity of the mutant hCBGs

To determine whether the replacement of Cys_{228} with serine or alanine results in an alteration of steroid binding properties, Scatchard analysis [15] was used to compare the [^{3}H]cortisol binding affinities of wild-type and mutant hCBGs. Within experimental error, all of these species exhibit an identical K_d for cortisol ($1–2 \times 10^{-9}$ M at pH 8.0, 4°C). These findings strongly suggest that the SH moiety of Cys_{228} does not play a direct role in steroid binding.

Effect of SH-specific reagents on the steroid binding activities of wild-type and mutant hCBGs

The production of biologically active mutant hCBGs containing only one cysteine (Cys_{60}) allowed us to investigate the role of this residue in steroid binding. For this purpose, the mutant and wild-type proteins were modified with SH-specific reagents, and the effects on steroid binding were measured. First, serum-free medium derived from infected *Sf 9* cells was concentrated several fold and buffer exchanged so that the final pH was 8.0. Samples were then treated with 200 mM *N*-ethylmaleimide (NEM) for 30 min, with or without prior treatment with 50 mM DTT. In the case of DTT pretreatment, DTT was removed before exposure to NEM. After NEM treatment, excess

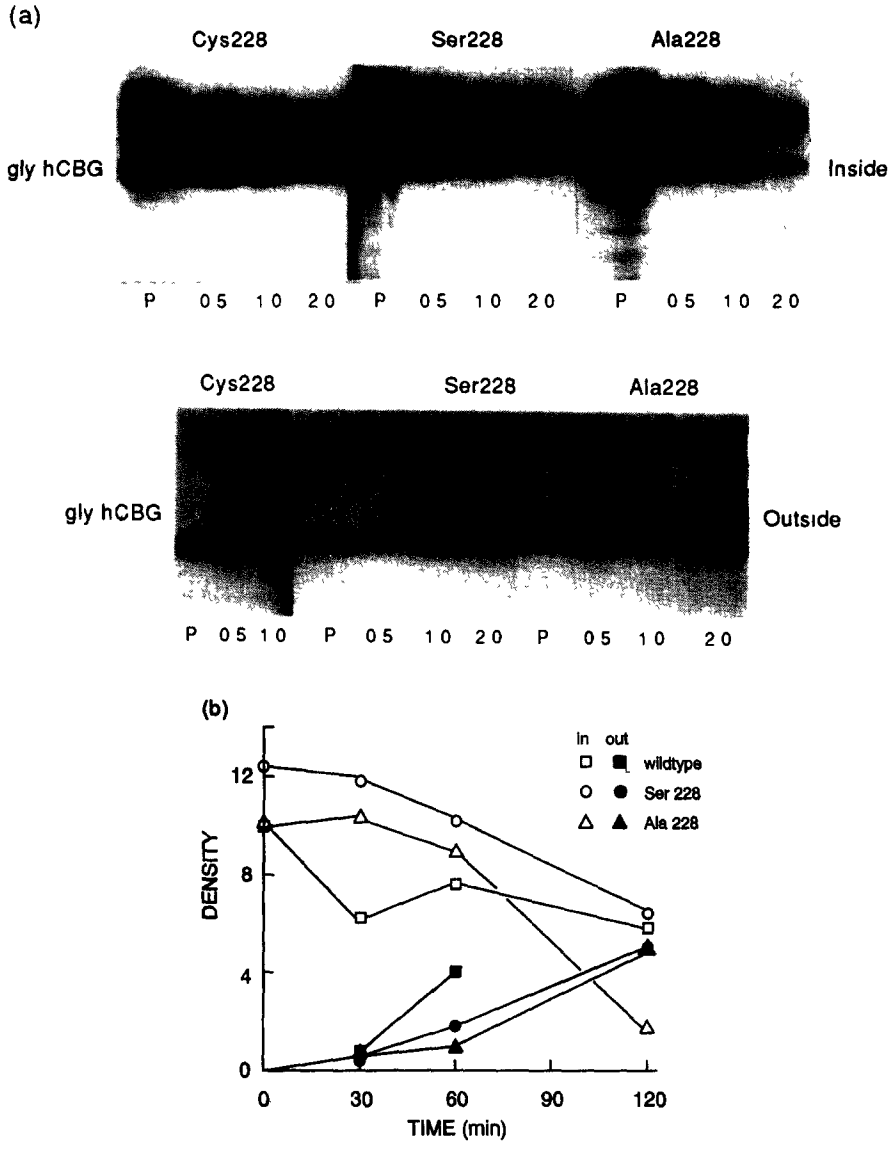

Fig. 2. Time course of synthesis and secretion of wild-type and mutant ($Cys_{228}\rightarrow Ser_{228}$; $Cys_{228}\rightarrow Ala_{228}$) hCBGs. *Sf9* cells infected with recombinant baculovirus encoding wild-type or mutant hCBGs were pulse-labeled for 1 h with [^{35}S]methionine (100 μCi/ml), and chased in the presence of 10 mM unlabeled methionine for the indicated times (0.5, 1, and 2 h). Cells and media were harvested at each time and subjected to immunoprecipitation with anti-hCBG antibodies. (a) SDS–PAGE resolution of immunoprecipitates of the cell lysates (inside) and medium samples (outside). (b) Quantitation by densitometry.

reagents were removed by passing the reaction mixture through a spin column of Sephadex G-25, after which cortisol binding was measured for this sample.

NEM treatment in the absence of DTT results in ~60 and ~40% inhibition of [^{3}H]cortisol binding by the wild-type hCBG and hCBG–Ala_{228}, respectively (Table 1). Pretreatment of these proteins with DTT increased the inhibition to ~80 and ~90%, respectively, suggesting that there is partial oxidation of the cysteine in the expressed protein. hCBG–Ser_{228} shows similar NEM sensitivity (data not shown). In all cases, inactivation occurs within 10 min of incubation with NEM. These results suggest that the SH moiety of Cys_{60} is either directly involved in steroid interaction or is important for maintaining the structure of a functional steroid binding site. Alternatively, it is possible that the inactivation results from steric hindrance produced by chemical modification of the SH group at the steroid binding site.

The wild-type and mutant proteins were also treated with 100 mM iodoacetamide (IAA) using a similar protocol. This treatment was more effective than NEM in eliminating the cortisol binding activity of wild-type and mutant hCBGs (Table 1), and it does not require pretreatment with DTT. Kinetic analysis showed that IAA inactivation is complete within 30 s of treatment (data not shown). This inactivation occurs at pH 8.0 or above, consistent with modification of a cysteine residue in the steroid binding site. Inactivation by IAA does not require prior reduction of wild-type and mutant proteins. The fact that prior reduction is not required to achieve complete inactivation by IAA, may be explained by the high reactivity of IAA (since

Table 1. Effect of SH reagents on the cortisol-binding activity of wild-type and mutant hCBGs

Treatment	Wild-type cpm	Wild-type Residual activity (%)	Ala228 cpm	Ala228 Residual activity (%)	Ser228 cpm	Ser228 Residual activity (%)
IAA						
Control	2730		2347		650	
100 mM	34	1	110	5	37	6
NEM						
Control	4200		1794			
200 mM	1523	36	1012	56		
(Pretreat with 50 mM DTT)						
Control	1551		1012			
200 mM	259	17	83	8		
$Na_2S_4O_6$ (pretreat with 50 mM DTT)						
Control	9490		4132		1110	
100 mM	198	2	450	10	301	27

Cortisol binding activity was assessed by a filter assay using [^{3}H]cortisol [11]. The differences in cpm of the different samples prior to treatment with a particular SH reagent reflect differences in their respective protein concentrations. Samples pretreated with DTT were passed through a second G-25 spin column to remove excess DTT before subsequent treatment with NEM. Control experiments showed that under the assay conditions (saturating steroid) 10 mM DTT did not significantly inhibit steroid binding.

iodine is a good leaving group), what may allow it to react with a partially oxidized SH group.

The wild-type and mutant hCBGs were also treated with another SH-specific reagent, sodium tetrathionate, in a similar experimental protocol. Within 5 min of treatment with 100 mM sodium tetrathionate, cortisol binding activity is effectively abolished. This result confirms that it is the SH group of Cys_{60} that is in the steroid binding site (Table 1).

DISCUSSION

Affinity labeling and chemical modification studies on hCBG, purified from human serum, suggested that one or both of the two cysteine residues in this protein are in the immediate vicinity of the steroid binding site and may be directly involved in steroid–protein interaction [2, 16]. Le Gaillard and Dautrevaux [17] reported that one cysteine is more accessible than the other, and that modification of the more accessible SH group with ethyleneimine did not induce any loss of binding activity in hCBG. This suggested that the inaccessible cysteine might be buried within the steroid binding site. Comparison of the cDNA sequences of human, rat, and rabbit CBG reveals that only Cys_{228} is conserved in evolution [3–5], and this residue is present within a conserved flanking sequence of 40 amino acids [6]. The other cysteine residue in hCBG, Cys_{60}, is replaced by a serine in rabbit [5] and by an alanine in rat [4]. Moreover, Cys_{228} is the only cysteine residue in the rat protein [4]. Thus, it was our expectation that Cys_{228} would be the inaccessible cysteine which is critical for steroid binding in hCBG.

To ask whether Cys_{228} is indeed involved in steroid binding, we used site-directed mutagenesis to create hCBG cDNAs in which the codon for Cys_{228} encodes serine or alanine. Serine was chosen because it is chemically and sterically similar to cysteine. If the cysteine SH group is directly involved in hydrogen bonding with some part of the steroid molecule, we predicted that this interaction would be maintained in the serine mutant, which should retain that hydrogen bonding possibility. However, in the alanine mutant, we expected that this kind of interaction should be abolished, since the side chain of alanine has a methyl group that would eliminate the possibility of hydrogen bond formation.

To compare the properties of the wild-type and mutant proteins, we produced these proteins in baculovirus-infected insect cells. The rates of synthesis and secretion of the mutant hCBGs are very similar to those of the wild-type protein (Fig. 2). Furthermore, the spectrum of molecular forms (representing differentially glycosylated species) is also identical among these proteins, suggesting that the posttranslational modifications of these mutants are identical in the mutant and wild-type polypeptides. We conclude that these single amino acid substitutions cause no major change in either protein biogenesis or overall conformation.

Scatchard analysis of steroid binding data obtained from the filter assay shows that both mutant hCBGs have a high affinity for cortisol, identical with that of wild-type hCBG. On the one hand, if Cys_{228} is involved in steroid binding, we might predict that hCBG–Ser_{228} could retain cortisol binding activity because serine is chemically and structurally a conservative mutation. In

this case, we would expect a decrease in affinity, since the OH of serine is a weaker nucleophile than the SH group of cysteine; however, no change in affinity was observed. On the other hand, the demonstration that hCBG–Ala_{228} is equally active argues against Cys_{228} being the functionally important cysteine since in this mutant the hydrogen bonding ability of residue 228 has been eliminated. This led us to the conclusion that Cys_{60}, rather than Cys_{228}, is the cysteine residue involved in steroid binding.

Although the previous investigations pointed to a buried or inaccessible cysteine residue within the steroid binding site [16–18], the present work provides evidence that the accessible cysteine is functionally important. For example, we find that after treatment with NEM, the binding activities of both the wild-type and the mutant proteins are significantly reduced (Table 1). The inactivation of steroid binding by IAA, which has a pH optimum around 8.0, further suggests that an accessible SH group is involved in steroid binding, since the pK_a of the cysteine SH group is around 8.0. Moreover, the observation that the kinetics of this inactivation are very rapid (30 s for 100% inactivation) eliminates histidine as the target for modification, since the rate of histidine modification by IAA is very slow [13]. To provide further confirmation that a cysteine is the residue modified in the above experiments, we tested the effect of sodium tetrathionate which reversibly modifies SH groups [14]. Sodium tetrathionate also completely abolished the activity of both wild-type and mutant hCBGs.

In conclusion, site-directed mutagensis coupled with specific chemical modification unambiguously demonstrate that Cys_{228} is functionally unimportant. The data support but do not prove the hypothesis that the SH group of Cys_{60} is directly involved in the steroid–protein interaction in hCBG (at a minimum, this residue must be in close proximity to the bound steroid molecule). The fact that the side chains of Cys_{60} and Cys_{228} do not form a disulfide bridge [16] suggests that the two cysteines residues are not immediately adjacent in the tertiary structure. Nevertheless, they could be sufficiently close that chemical modification of one could sterically preclude modification of the other, as observed by Le Gaillard and Dautrevaux [17]. In addition, the fact that a cysteine forms a covalent adduct with 6β-bromoprogesterone [3] does not necessarily require that the residue is essential for steroid binding. Instead, it is consistent with the general conclusion that one of the cysteines must be associated with the steroid binding site [16]. Therefore, an interpretation of our data that is consistent with the general literature, is that both Cys_{228} and Cys_{60} reside in or near the steroid binding site, and it is possible that neither cysteine *per se* is required for steroid binding.

Further mutagenesis and chemical modification experiments are in progress to determine whether the SH group of Cys_{60} is directly involved in the steroid–protein interaction or whether chemical modification simply blocks access to the steroid binding site. In addition, the single cysteine hCBG mutants provide us a basis for higher resolution investigation by use of SH-specific probes in conjunction with spectroscopic methods.

Acknowledgements—The authors thank Dr Gerald Schwartz and Dr Ronald Kohanski for suggestions and advice regarding specific chemical modification of cysteine residues, and we thank Dr Arthur Cederbaum and coworkers for sharing their tissue culture facilities This work was supported by NIH Grants GM-38501 (R.G) and GM-39750 (J.B.A.R).

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