Corticosteroid-binding globulin (CBG) is a non-inhibitory serine proteinase inhibitor (serpin) that transports cortisol and progesterone in blood. Crystal structures of rat CBG and a thrombin-cleaved human CBG:antitrypsin (Pittsburgh) chimaera show how structural transitions after proteolytic cleavage of the CBG reactive centre loop (RCL) could disrupt steroid-binding. This ligand-release mechanism is assumed to involve insertion of the cleaved RCL into the β-sheet A of the serpin structure. We have therefore examined how amino acid substitutions in the human CBG RCL influence steroid-binding before and after its cleavage by neutrophil elastase. Elastase-cleaved wild-type CBG or variants with substitutions at P15 and/or P16 (E334G+G335N or E334A) lost steroid-binding completely, while deletion of E334 resulted in no loss of steroid-binding after RCL cleavage, presumably because this prevents its insertion into β-sheet A. Similarly, the steroid-binding properties of CBG variants with substitutions at P15 (G335P), P14 (V336R) or P12 (T338P) in the RCL hinge were largely unaffected after elastase-cleavage, most likely because the re-orientation and/or insertion of the cleaved RCL was blocked. Substitutions at P10 (G340P, G340S) or P8 (T342P, T342N) resulted in a partial loss of steroid-binding after proteolysis, which we attribute to incomplete insertion of the cleaved RCL. Remarkably, several substitutions (E334A, V336R, G340S, and T342P) increased the steroid-binding affinities of human CBG, even prior to elastase cleavage, consistent with the concept that CBG normally toggles between a high-affinity ligand-binding state where the RCL is fully exposed, and a lower-affinity state in which the RCL is partly inserted into β-sheet A.
serpin-proteinase complex. Intriguingly, the RCLs are perhaps the most poorly conserved regions of individual serpins with the same function (6), and the reasons for this are not fully understood.

It has been proposed that the serpin structure has been adapted by CBG and the closely-related thyroxin-binding globulin (TBG) to control hormone release at their sites of action after the RCL is cleaved by proteinases (9-12). This is based on evidence that the S→R conformational transition of CBG is accompanied by a marked increase in thermostability (9) and decrease in steroid-binding affinity (9,10) following cleavage by neutrophil elastase. Recently we showed the 1.9 Å crystal structure of cortisol-bound rat CBG displays a typical native serpin conformation, with the RCL fully exposed from the β-sheet A (13).

The crystal structure of a thrombin-cleaved human CBG-AAT chimaera has since been reported, in which the RCL of human CBG was replaced with a AAT (Pittsburg) variant sequence (14), presumably to facilitate cleavage by thrombin. In this structure, the cleaved AAT RCL was fully incorporated into the β-sheet A of CBG, and soaking the crystals in cortisol led to the surprising observation that the cortisol-binding site of the cleaved CBG-AAT chimera adopts a conformation that resembles that of native CBG (14). However, elastase cleavage of CBG results in an irreversible loss of steroid-binding, that cannot be restored by adding an excess of steroid ligand (9,10), and crystal packing effects and high ligand soaking concentrations might have allowed the binding of cortisol with low affinity to the cleaved CBG-AAT chimera. Moreover, the effect of replacing the CBG RCL with the corresponding AAT (Pittsburgh) sequence may itself have influenced steroid-binding because previous studies have indicated that human thyroxin-binding globulin (TBG)-AAT chimaeras containing various AAT RCL sequences have either increased or reduced ligand-binding affinities (15). It should also be noted that the proteolytic cleavage site (P1-P1') in human AAT (16) differs in location to that in human CBG, i.e., Val344-Thr345, which correspond to the P6 and P5 residues in the AAT RCL (9). In other words, the cleaved RCL of human CBG is five residues shorter than that of AAT or the AAT (Pittsburgh) variant, and thus the composition and length of the cleaved RCL in the thrombin-cleaved human CBG-AAT chimera structure (14) are likely quite different from those in normal human CBG.

Analyses of the human CBG-AAT chimera structure (14) also led to the conclusion that a flip-flop movement of the intact RCL into and out of β-sheet A allows the equilibrated release of cortisol, but direct evidence for this is lacking. To address the issue in a different way, we have substituted specific residues within the RCL of human CBG and determined how this influences its proteolytic cleavage by neutrophil elastase in relation to its cortisol-binding properties. This has not only enabled us to identify key residues within the RCL that control the S→R transition of CBG which results in hormone release, but has provided evidence that partial insertion of the intact RCL within the human CBG β-sheet A exerts an allosteric effect on its cortisol-binding activity.

EXPERIMENTAL PROCEDURES

Cell culture - Chinese hamster ovary (CHO) cells were maintained in α-minimum essential medium (Invitrogen Canada Inc., Burlington, Ontario, Canada) containing 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C with 5% CO_2.

Human CBG constructs and site-directed mutagenesis - A full-length cDNA encoding the human CBG precursor (4,17) was subcloned into a HindIII/XbaI-digested eukaryotic expression vector pRe/CMV (Invitrogen). All mutations were performed on the wild-type human CBG expression vector template with the QuickChange II XL Site-Directed Mutagenesis kit (Stratagene Inc., La Jolla, CA) according to the manufacturer’s instructions. The forward and reverse primers used for mutagenesis are summarized in Table 1. All constructs were sequenced to confirm that only the targeted mutations had occurred.

Expression of wild-type and variant human CBGs - To establish cell lines expressing wild-type or variant human CBGs, CHO cells were cultured at 60% confluence in 6-well dishes and then transfected with 2 μg of expression plasmids using Lipofectamine2000 (Invitrogen) as suggested by the manufacturer. One day later, cells were sub-cultured at a dilution of 1:10 and selected in the presence of 1.5 mg/ml neomycin (Invitrogen) for one week. Stably transfected CHO cells were then grown to near confluence. After washing twice with phosphate-buffered saline, the cells were cultured for 4 days in phenol red-free, high-glucose DMEM medium (Invitrogen) containing 100 nM cortisol. The culture media were collected, dialyzed in a buffer containing 20 mM Tris (pH 8.0) and 100 nM cortisol at 4 °C overnight, and subjected to ion exchange chromatography on a Mono Q fast protein liquid chromatography column (GE Healthcare Bio-Sciences, Chandler, AZ) to semi-purify the recombinant CBGs for functional analysis.
Elastase digestion - Human neutrophil elastase (Elastin products Co., Inc., Owensville, MO) was reconstituted in 0.05 M NaAc (pH 5.0) and 0.1 M NaCl. Unless otherwise stated, elastase digestions were performed with 1 μg of semi-purified CBGs and 0.05 μg of elastase for 5 min at 37 °C, as described previously (10). Reaction products were analyzed by Western blotting after reduction by boiling in the presence of 0.1 M β-mercaptoethanol and 1% sodium dodecyl sulfate (SDS). Human neutrophil elastase typically cleaves human CBG in a single location, and reduces its apparent molecular mass by 5 kDa which can be resolved by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), as described previously (10).

Western blot analysis - Proteins were resolved by SDS-PAGE (5% stacking and 10% separating gels) and transferred to a nitrocellulose membrane. After incubation with blocking buffer (5% fat-free milk in PBS containing 0.1% Tween 20), blots were incubated with rabbit anti-human CBG antisera at a 1:1,000 dilution (18). Immuno-reactive proteins were further detected with an HRP-labeled goat anti-rabbit IgG (Sigma-Aldrich Canada Ltd., Oakville, Ontario, Canada) and chemi-luminescent substrates (Pierce, Rockford, IL) by exposure to x-ray film.

Steroid-binding analyses - [1,2-3H]Cortisol (55 Ci/mmol) was purchased from PerkinElmer Life Sciences (Boston, MA). Unlabeled steroids were purchased from Sigma-Aldrich. The steroid-binding capacities of equivalent amounts of immuno-reactive CBGs, determined by Western blotting, were then assayed by a routine saturation analysis employing [3H]cortisol as the labeled ligand and dextran-coated charcoal (DCC) as a separation agent (19).

To compare the steroid-binding capacities of CBG before and after elastase cleavage, purified protein was first stripped of endogenous steroid by incubation with DCC for 30 min at room temperature, and then saturated with an excess of [3H]cortisol for 1 h at room temperature. Half of the [3H]cortisol-saturated proteins were then subjected to elastase cleavage, as described above, while the remainder was held under the same conditions without addition of elastase. Aliquots of cleaved and un-cleaved CBG-[3H]cortisol complexes were subjected to Western blot analysis, and the rest was rapidly chilled and used to measure the amount of bound [3H]cortisol in samples after absorption of free steroid with DCC for 10 min at 0 °C (19). The apparent dissociation rates of CBG-bound [3H]cortisol were also assessed by the exposure of the cleaved or non-treated [3H]cortisol-saturated sample to DCC for different time intervals at 0 °C (19). In addition, equilibrium binding constants of untreated wild-type CBG and the CBG variants were determined by Scatchard analysis, using [3H]cortisol as the labeled ligand (19).

RESULTS

Amino acid substitutions at P16 and P15 in the human CBG RCL type I β-turn – Alignment of the CBG sequences from different mammalian species, including human (NCBI accession code NP_001747), rat (NP_001009663), mouse (NP_031644), rabbit (P23775), pig (AAG45431), and sheep (CAA52000), against human AAT (NP_000286), shows that their RCL sequences are hyper-variable (Fig. 1A). The numbering of residues in the RCL is generally made in relation to the P1-P1´ (Met-Ser) proteinase cleavage site of human AAT (Fig. 1A). Although the normal elastase cleavage sites of human CBG and AAT are in quite different locations within their RCLs (Fig. 1A), neutrophil elastase will cleave AAT in almost the same relative (P6-P7) location as the CBG cleavage site at P5-P6 (Fig. 1A), if the Met residue at P1 in human AAT is oxidized (20). The amino acid substitutions we have made within the CBG RCL (Fig. 1B) are also shown in relation to the P1-P1´ cleavage site in AAT together with a model of how the cleaved CBG RCL is thought to reposition itself between the β-strands s5A and s3A.

We first examined the effects of several substitutions within the type I β-turn of the RCL (residues 332 to 335), and focused our attention on E334 and G335 in human CBG to determine whether they represent critical elements of the hinge that allows repositioning of the RCL after proteinase cleavage (Fig. 1B). The amino-terminus of the CBG RCL is not well conserved across species, and in human CBG is represented by E334 at P16 (Fig. 1A). By contrast, the G335 at P15 in human CBG is conserved in mammalian species apart from rodents (Fig. 1A). However, it has been suggested that the glycine residue at P16 in rodent CBGs plays an equivalent role to G335 in human CBG in allowing the RCL to reposition itself after cleavage (13). We therefore first converted the P16 (E334) and P15 (G335) residues of the human CBG RCL into the corresponding residues in rat CBG, i.e., G334 and N335, and tested this mutant for its ability to be cleaved by neutrophil elastase, and to bind steroid before and after elastase cleavage. As in the case of wild-type CBG, it is evident that these amino acid substitutions do not limit elastase cleavage, as assessed by Western blotting (Fig. 2A), or influence the loss of steroid-binding capacity that typically occurs after proteolysis of the RCL of human CBG.
(Fig. 2B). The Western blotting data (Fig. 2A) show the typical reduction in the apparent size of human CBG by about 5 kDa, consistent with the loss of the 39 carboxyl-terminal residues that are not recognized by our antibodies after cleavage by elastase (21).

The steroid-binding activities of various CBG mutants were also compared with wild-type CBG in a simple assay to assess the apparent rate of [3H]cortisol dissociation from their steroid-binding sites before and after treatment with elastase (see Experimental Procedures for details). Although this method provides an indirect measure of binding affinity, it can be performed immediately after elastase treatment thereby circumventing further proteolysis of CBG beyond cleavage of the RCL, which can be monitored in parallel by Western blotting (Fig. 2A).

In the cases of wild-type CBG and the E334G+G335N human CBG variant, elastase cleavage clearly results in a substantial (5 - 10 fold) reduction in the amount of [3H]cortisol bound to the proteins (Fig. 3 left panels of A and B). The small amount of specifically-bound [3H]cortisol that remains after the 5 min elastase treatment likely represents the sum of (a) the steroid-binding of residual un-cleaved proteins, and (b) the contribution made by the relative large amounts of cleaved proteins with much reduced binding affinity. To assess this further, we expressed the bound [3H]cortisol at each time-point of DCC exposure as a percentage of the extrapolated values at the zero time point, and this shows that the dissociation of [3H]cortisol from these proteins after elastase cleavage is only about 50% faster than that measured for the intact proteins (Fig. 3 right panels of A and B). We therefore conclude that the bound [3H]cortisol, remaining after elastase cleavage, primarily reflects binding to a small amount of intact proteins, plus a minor contribution from the cleaved proteins from which [3H]cortisol dissociates very much more rapidly, and which would be very difficult to accurately measure.

To assess the individual contributions of E334 and G335 to the loss of steroid binding from the E334G+G335N CBG variant after elastase cleavage, we first focused on E334 and either deleted it or substituted it with an alanine residue. Since the glutamic acid (E333) at P17 in human CBG is highly conserved in serpin structures (Fig. 1A), deletion of E334 would mean that the G335 would still be adjacent to the P17 glutamic acid, as it is in rat CBG (Fig. 1A). The loss of E334 had no impact on the cleavage of CBG by elastase (Fig. 2A), but surprisingly its binding capacity after elastase cleavage was essentially unchanged (Fig. 2B). This was confirmed in our assay of the apparent dissociation [3H]cortisol from its binding site, when expressed either in terms of the actual amounts (cpm) of bound [3H]cortisol (Fig. 3C left panel) or a percentage of the bound counts remaining at set time points after DCC exposure (Fig. 3C right panel). By contrast, when the E334 in human CBG was substituted with an alanine residue, and this variant was cleaved by elastase (Fig. 2A), it clearly behaved like the wild-type protein and lost about 80% of its [3H]cortisol binding capacity (Figs. 2B and 3D).

We then replaced the P15 G335 with proline to stiffen the segment of the RCL that is tethered to s5B, since the reduced flexibility should greatly impair conformational changes in this region during RCL insertion. When this G335P CBG variant was examined, we found that it retained its steroid-binding activity after proteolytic cleavage (Fig. 2A and 2B), and this was confirmed in our dissociation-rate assay (Fig. 3E). In Western blotting experiments, the G335P variant and two other variants (T338P+A339N and G340P) were consistently less immuno-reactive after elastase cleavage, without any evidence of more extensive proteolysis, and we attribute this to the loss of an epitope within the cleaned RCL.

Amino acid substitutions at P14 and P12 of the CBG RCL have profound effects on steroid-binding – When we replaced the V336 at P14 of human CBG with a large, charged residue (arginine), this did not influence its cleavage by elastase (Fig. 4A). Moreover, cleavage of the V336R variant did not reduce its steroid-binding capacity (Fig. 4B) or the rate of [3H]cortisol dissociation from its binding site (Fig. 5A). We also substituted the Thr338 at P12 with a proline alone or together with an A339N substitution at P11, because this would then introduce an N-glycosylation site, as found in the corresponding position within the rat CBG RCL (Fig. 1A). As in the case of the V336R CBG variant, the T338P and T338P+A339N substitutions had no effect on the proteolytic cleavage of the RCL by elastase (Fig. 4A), and their steroid-binding capacities appeared to even increase after elastase cleavage (Fig. 4B). In the case of the T338P and T338P+A339N human CBG variants, the latter observation was confirmed in our analysis of the rate of [3H]cortisol dissociation from their binding sites before and after elastase cleavage (Fig. 5B and C, respectively). However, in these cases it appears that the rate of [3H]cortisol dissociation from the elastase-cleaved proteins is slightly more rapid than from their intact counterparts (Fig. 5B and C).

Human CBG variants with substitutions at P10 and P8 in the RCL only partially loose steroid-binding after elastase cleavage – When we replaced Gly340
at P10 with a proline or serine (the corresponding rat CBG residue), and Thr342 at P8 with proline or Asn (the corresponding residue in rat CBG), the cortisol-binding capacities of these four variants (G340P, G340S, T342P, and T342N) were all partially reduced after elastase cleavage (Fig. 6). To further assess this, the apparent dissociation rates of [3H]cortisol from the native and elastase-cleaved forms of these variants were measured. As shown in Fig. 7, the [3H]cortisol-binding capacities of the G340P, G340S, and T342N CBG variants were reduced (left panel), and their [3H]cortisol dissociation rates were clearly increased (right panel) after elastase cleavage. Thus, indicating that the steroid-binding properties of these CBG variants are not completely disrupted when their RCLs are cleaved.

We also noticed that the T342P variant appeared to be incompletely cleaved by elastase at the 5 min time point routinely used in our assays (Fig. 6A). Since this residue is closest to the elastase-cleavage site in the CBG RCL, we assumed that it might hamper the proteolytic reaction. To explore this, we performed the elastase digestion for 5, 15, 30, and 60 min, and found that samples incubated for 15, 30, and 60 min were more completely cleaved when compared to the 5 min digestion (Fig. 8A). More importantly, the loss in cortisol-binding capacity of the T342P variant also decreased progressively between the 5 and 60 min treatments with elastase (Fig. 8B). We therefore compared the steroid-binding properties of the T342P variant before and during progressive exposures to elastase, and observed a progressive increase in the apparent rate of [3H]cortisol dissociation from the elastase-cleaved protein with increased time of proteolysis (Fig. 8C).

Substitutions in the RCL of human CBG that increase steroid-binding affinity – To assess whether the amino acid substitutions we introduced into the RCL of human CBG have any impact on the steroid-binding properties of the native proteins, i.e. prior to elastase cleavage, we compared them with wild-type CBG in the [3H]cortisol dissociation-rate screening assay, as well as by Scatchard analysis. Surprisingly, while the affinities of most CBG variants for [3H]cortisol were indistinguishable from that of wild-type CBG in these assays, it was apparent that [3H]cortisol dissociated more slowly from the E334A, V336R, G340S, and T342P variants (Fig. 9A). This suggested that these variants have a higher inherent affinity for cortisol when compared to the wild-type protein, and this was confirmed by Scatchard analysis (Fig. 9B), which demonstrated that these four CBG variants, bind [3H]cortisol with higher affinities, with equilibrium binding constant (Kd) values of 0.90, 1.16, 1.00 and 1.10 nM, respectively, when compared to wild-type CBG (Kd = 1.56 nM).

DISCUSSION

It has been proposed that several regions within the RCL of serpins are involved in controlling the conformational changes that occur after interactions with their target proteinases: the hinge (P15–P9 portion of the RCL); the shuttle (top of β-sheet A); the breach (near the center of β-sheet A), and the gate (area including s3C and s4C) (8). Among these regions the hinge is thought to provide the essential mobility required for conformational changes in the RCL during its incorporation into the serpin β-sheet A structural elements after proteolysis (8,22). The RCL is hyper-variable in its composition and length, and in the case of inhibitory serpins this is presumed to reflect an evolutionary adaptation for optimal interactions with their target proteinases (8,23).

However, there is a general consensus in the hinge region sequences of inhibitory serpins, with P15 usually represented by glycine, P14 a non-charged residue mostly threonine or serine, and hydrophobic residues with short side-chains at P12 to P9 (6,8). Sequence comparisons of inhibitory and non-inhibitory serpins demonstrate that residues P12 to P9 in the inhibitory serpins have a >50% conservation of alanine at each position, and the permissible replacements are all relatively small residues, mostly valine, serine, threonine, or glycine, whereas non-inhibitory serpins have few or no alanines at these positions (6). In inhibitory serpins, the rate of RCL insertion relative to the cleavage reaction determines the efficiency of the inhibition (6). Therefore, the composition of the RCL, and particularly the residues that become buried, is a key determinant of the rate of loop insertion. Analysis of this segment in CBGs from different species shows that it has the overall characteristics necessary for this conformational transition and repositioning.

In most serpins, a conserved glutamic acid at P17 hydrogen bonds with a conserved threonine in s4C; forms a salt bridge to a conserved lysine in s6A, and packs against a conserved methionine in s3C (8). This tight packing of the P17 residue is functionally very important because naturally occurring mutations at P17 in AAT (antitrypsin Z) (24) and heparin cofactor II (25) disrupt the salt bridge, and cause these mutant proteins to polymerize. Thus, the P17 glutamic acid in serpin RCLs has an important stabilizing role, and it is clear that it limits the mobility of the β-turn because it is anchored in the same ways in both cleaved and un-cleaved serpins, as illustrated in the native rat CBG (13) and cleaved human CBG-AAT chimera (14) crystal structures. In
cleaved serpins, the N-terminus of s4A is tethered to strand s5A via a type I β-turn, which in human CBG includes the P16 (glutamic acid) and P15 (glycine) residues of the RCL (13). Our data showed that substitution of the P16 (E334) and P15 (G335) residues of human CBG with the corresponding residues (Gly and Asn) in rat CBG had no effect on the steroid-binding properties of this variant upon elastase cleavage, when compared to human wild-type CBG. This re-affirms our prediction that the glycine at P16 in rodent CBGs substitutes for the P15 glycine in other species, and the difference in the positioning of these glycines between species is unlikely to limit the repositioning of the cleaved rat CBG RCL as a new s4A β-sheet (13).

In parallel, we substituted the E334 at P16 in human CBG with alanine, and the G335 at P15 with proline. Although the E334A variant lost its cortisol-binding activity in the same way as wild-type CBG after elastase cleavage, the cortisol-binding properties of the G335P variant did not change either before or following elastase cleavage. This implies that the RCL of the G335P variant remains expelled even after it is cleaved, and reinforces the essential role that this glycine plays in the type I β-turn with respect to the repositioning of the cleaved RCL. Likewise, deletion of E334 in human CBG resulted in a variant that fully retains its steroid-binding activity after elastase cleavage, which again implies that its RCL remains expelled after elastase cleavage. However, it should also noted that, when the RCL normally integrates within the β-sheet A, the side chains of evenly numbered P residues (P14, P12, etc) point towards the core of the protein, while the side chains of the other residues in the cleaved RCL remain surface exposed (13). It is therefore possible that the deletion of E334 at the beginning of the RCL interferes not only with its reorientation, but alters its registration with respect to residues within the β-sheet A, and thereby disrupts its integration as a novel s4A β-strand.

The formation of inhibitory serpin-proteinase complexes results in full insertion of the RCL, which presses the proteinase to the bottom of the serpin (6). Thus, the rate of RCL insertion will determine the efficiency of the inhibitory pathway, and it is not surprising that a non-charged, small hydrophobic P14 residue plays an important role in initiating the RCL registration, since its side chain is the first to pack within the hydrophobic core of the cleaved serpin (6). The importance of P14 in affecting the rate of the loop insertion was first demonstrated by a variant of AAT that contains a large, charged arginine residue, in place of the small, neutral threonine at P14 (26), and was confirmed in studies of plasminogen activator inhibitor-1 variants in which the P14 threonine was replaced by a battery of different residues (27). In the latter study, the largest changes in the stoichiometry of inhibition and the rate constants for RCL insertion were caused by substitutions with charged amino acids, of which arginine caused the greatest effect (27). Moreover, the structure of a cleaved antichymotrypsin variant with a T345R substitution at P14 revealed that the R345 side chain is not buried, resulting in incomplete strand s4A insertion (28). Complementary studies on ovalbumin, a non-inhibitory serpin that has an arginine residue at P14 and lacks the loop insertion mechanism (29), indicate that when this P14 arginine (R339) in ovalbumin is substituted with serine, the variant is characterized by an increased RCL insertion-rate and spontaneous partial insertion of the cleaved RCL (30). Moreover, the crystal structure of the cleaved R339T ovalbumin variant clearly revealed that complete loop insertion can occur upon this single hinge mutation (31). These reports are all very much in line with our finding that substitution of the P14 valine (V336) in human CBG with arginine does not hinder cleavage of its RCL by elastase or alter its steroid-binding properties after cleavage, presumably because the arginine at P14 prevents the re-orientation and/or subsequent insertion of the cleaved RCL.

In the present study, we replaced the P12 T338 in human CBG with a proline (T338P), as in rat CBG (Fig. 1A). Moreover, since rat CBG has a unique N-glycosylation site (N-S-T) in the position corresponding to P11-P9 in human CBG, we also substituted the alanine at P11 in human CBG with an aspartic acid in addition to the T338P substitution, thus creating a T338P+A339N human CBG variant that would also have a glycosylation consensus sequence (N-G-S) in the P11-P9 position. Our analyses of these variants showed that they are both effectively cleaved by neutrophil elastase, but that this does not reduce their steroid-binding activities.

Western blots of elastase-digested T338P+A339N human CBG, indicate that it is less immuno-reactive than most other elastase cleaved CBG variants. As in the case of the G335P variant, we attribute this to a loss antibody recognition rather than further proteolysis because the steroid-binding properties of the T338P+A339N variant are not lost, and even appear to be enhanced after RCL cleavage. Thus, although the presence of an N-glycosylation site within the RCL does not hamper its cleavage by elastase, our data suggest that substitutions at P11 and P12 of human CBG, which were introduced to reflect the rat CBG sequence, appear to block RCL insertion after elastase cleavage. The fact that the T338P substitution was sufficient to cause this effect raises the intriguing question of whether CBG RCL
sequences have evolved in parallel with species-specific differences within β-sheet A in order to accommodate insertion of the cleaved RCL, rather than simply being an evolutionary adaptation to allow optimal recognition of their RCLs by species-specific proteinases (23). In addition, although we recognize that a proline is present at P12 in other serpins (6), as well as in rat CBG, further studies are warranted to determine if and how the cleaved RCL of rat CBG inserts within β-sheet A.

In addition to P12, the P10 residues in serpin RCLs appear to play critical roles in the inhibitory functions of serpins, as illustrated by the deficient inhibitory properties of naturally occurring variants of antithrombin and C1 inhibitor (6). It has also been reported that substitution of G349 at P10 in AAT to proline alters its inhibitory properties (22). Since the P10 and P8 residues in human CBG (G340 and T342, respectively) are much closer to the elastase cleavage site of human CBG than in AAT, we replaced them with either a proline or the corresponding residues in the rat CBG (Fig. 1A). The results of these experiments are of interest, because the steroid-binding affinities of these variants are partially preserved, even after what appears to be complete RCL cleavage, and this is most obvious with respect to the G340P variant. This suggests that their RCLs are only partly inserted after elastase cleavage. Perhaps of greater interest is the observation that two of these variants share an interesting property with the E334A and V336R variants in that their affinities for cortisol are actually higher than those of wild-type human CBG, even before elastase treatment, thus suggesting that the conformation or position of their un-cleaved RCL somehow influences the steroid-binding site. This observation supports the concept that the RCL of CBG normally toggles between two conformational states (13): a steroid-bound state in which the RCL is fully exposed, and an un-ligated state in which the RCL is partially inserted, as observed for the ligand-dependent repositioning of the heparin cofactor II (32) and anti-thrombin (33) RCLs. If this indeed occurs, our data would be consistent with concept that a fully exposed RCL, as most likely exists at least in the cases of the E334A and V336R variants, would result in CBG molecules with constitutively higher steroid-binding affinities because the RCL cannot toggle between the two conformational states (Fig. 10).

Our data also support the assumption that once the first and most critical P14 residue has inserted after RCL cleavage, the subsequent residues insert with increasing ease, at least until the F-helix is encountered at about P9 (6). In most serpins, P8 is represented by a threonine with a small side chain (6). From the crystal structure of TBG it has been suggested that the proline at P8 limits loop insertion within the upper half of the β-sheet A, and that in most serpins the barrier formed by a highly conserved histidine, in the lower halves of strand s3A and s5A, is broken by the side chain of a P8 threonine upon entry of the loop (34). Although there is no direct evidence for this, our results support it because substituting the P8 threonine in human CBG with proline appears to result in limited loop insertion upon cleavage.

The unique S→R conformational transition characteristic of many serpins is critical to their functions, and serpinopathies have been linked to mutations in important regions such as the shutter, breach, and hinge of their RCLs (5). As mentioned above, amino acid substitutions in the RCL hinge regions of some serpins interfere with their inhibitory activity, and naturally occurring mutations within serpin RCLs have been linked to disease, such as at P10 of antithrombin III (35-37), as well as at P10 (38), P12 (39), and P14 (40) of C1-inhibitor. The rat CBG crystal structure (13) has revealed how several naturally occurring CBG variants could account for defects in steroid-binding (13), including human CBG variants L93H (41,42) located in helix D, and D367N(43) located in strand s4B, as well as the rat CBG variant M276I (44) in strand s6A, and the mouse CBG variant K201E (45) in strand s2C. Almost all of these naturally occurring CBG mutations were identified initially because they had low steroid-binding affinities, and our data raise the interesting possibility that other CBG variants exist with abnormally high-affinity steroid-binding sites that retain their activities after elastase cleavage of the RCL.

In summary, we have unambiguously defined the functional link between the proteolytic cleavage of the human CBG RCL and the disruption of its steroid-binding site. In particular we have revealed a crucial role for hinge residues at P15, P14, P12, P10 and P8 during insertion of the cleaved RCL as part of the typical S→R conformational transition that occurs in other serpins. Moreover, our data further support the hypothesis that human CBG exists in different conformational states with different steroid-binding affinities, both before and after cleavage by one of its key target proteinases, neutrophil elastase.
REFERENCES

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The abbreviations used are: CBG, corticosteroid-binding globulin; TBG, thyroxin-binding globulin; AAT, α1-antitrypsin; ACT, α1-antichymotrypsin; RCL, reactive center loop; S→R, stressed to relaxed.
FIGURE 1. Mutations in the reactive center loop (RCL) region of human CBG. A, multiple sequence alignment of RCL from human, rat, mouse, rabbit, pig and sheep CBG, against human AAT. The known proteinase cleavage sites are marked by blue boxes. The proteinase cleavage site in human AAT is commonly marked as P1 and P1’. Human neutrophil elastase cleaves human CBG at Val334 (P6) and Thr335 (P5). B, model of the RCL inserted as a novel β-strand (s4A) into β-sheet A (β-strands s2A - s6A) of human CBG after elastase cleavage, showing the amino acid substitutions examined in the present study. This model was drawn by the PYMOL (http://pymol.sourceforge.net) program using the cleaved human CBG:AAT chimera structure (2VDX).

FIGURE 2. Impact of amino acid substitutions at P16 E334 and P15 G335 of the human CBG RCL on cortisol-binding capacities after elastase cleavage. A, Western blot analysis of wild-type (WT) and variant human CBGs (E334G+G335N, E334del, E334A, G335P) treated for 5 min at 37 °C in the presence (+) or absence (-) of neutrophil elastase. B, aliquots of the corresponding CBG-[3H]cortisol samples used in A were taken to confirm the cortisol-binding capacities after elastase cleavage with the values obtained for the samples that were not treated with elastase (set at 100%). Assays were repeated 3-4 times in duplicate and the data are presented as means ± S.D.

FIGURE 3. Dissociation of [3H]cortisol from untreated and elastase-cleaved human CBGs. Dissociation of [3H]cortisol from CBGs (untreated or elastase-cleaved) was measured by incubating [3H]cortisol-saturated CBGs with dextran-coated charcoal (DCC) for increasing time (5, 10, 15 or 20 min) at 0 °C before centrifugation to separate CBG-bound from free [3H]cortisol. Aliquots of the corresponding samples were also run on a Western blot to confirm the cleavage by elastase, as illustrated in Figure 2A. A, wild-type (WT) CBG. B, E334G+G335N CBG. C, E334del CBG. D, E334A CBG. E, G335P CBG. The results are presented as CBG-bound [3H]cortisol in cpm (left panel) or the percentage of the CBG-bound [3H]cortisol at different times of DCC exposure in relation to the extrapolated CBG-bound [3H]cortisol at zero time of DCC exposure (right panel). Linear regression was used to fit the data, and the r values for the [3H]cortisol binding data were > 0.910.

FIGURE 4. Impact of amino acid substitutions at P14 V336, P12 T338, and P11 A339 of the human CBG RCL on cortisol-binding capacities after elastase cleavage. A, Western blot analysis of wild-type (WT) and variant human CBGs (V336R, T338P+A339N, T338P) treated for 5 min at 37 °C in the presence (+) or absence (-) of neutrophil elastase. B, aliquots of the corresponding samples used in A were taken for a cortisol-binding assay to compare their cortisol-binding capacities after elastase cleavage with the values obtained for the samples that were not treated with elastase (set at 100%). Assays were repeated 3-4 times in duplicate and the data are presented as means ± S.D.

FIGURE 5. Dissociation of [3H]cortisol from untreated and elastase-cleaved P14 V336, P12 T338, and P11 A339 human CBG variants. Dextran-coated charcoal (DCC) was added to [3H]cortisol-saturated CBGs (untreated or elastase-cleaved) at 0 °C, and the samples were incubated for 5, 10, 15 or 20 min at 0 °C before centrifugation to separate CBG-bound from free [3H]cortisol. Aliquots of the corresponding samples were also run on a Western blot to confirm the cleavage by elastase, as illustrated in Figure 4A. A, V336R CBG. B, T338P+A339N CBG. C, T338P CBG. The results are presented as CBG-bound [3H]cortisol in cpm (left panel) or the percentage of the CBG-bound [3H]cortisol at different times of DCC exposure in relation to the extrapolated CBG-bound [3H]cortisol at zero time of DCC exposure (right panel). Linear regression was used to fit the data, and the r values for the [3H]cortisol binding data were > 0.969.

FIGURE 6. Impact of amino acid substitutions at P10 G340 and P8 T342 of the human CBG RCL on cortisol-binding capacities after elastase cleavage. A, Western blot analysis of wild-type (WT) and variant human CBGs (G340P, G340S, T342P, T342N) treated for 5 min at 37 °C in the presence (+) or absence (-) of neutrophil elastase. B, aliquots of the corresponding samples used in A were taken for a cortisol-binding assay to compare their cortisol-binding capacities after elastase cleavage with the values obtained for the samples that were not treated with elastase (set at 100%). Assays were repeated 3-4 times in duplicate and the data are presented as means ± S.D.

FIGURE 7. Dissociation of [3H]cortisol from untreated and elastase-cleaved P10 G340 and P8 T342 human CBG variants. Dextran-coated charcoal (DCC) was added to [3H]cortisol-saturated CBGs (untreated or elastase-cleaved) at 0 °C, and the samples were incubated for 5, 10, 15 or 20 min at 0 °C before...
centrifugation to separate CBG-bound from free [3H]cortisol. Aliquots of the corresponding samples were also run on a Western blot to confirm the cleavage by elastase, as illustrated in Figure 6A. A, G340P CBG. B, G340S CBG. C, T342N CBG. The results are presented as CBG-bound [3H]cortisol in cpm (left panel) or the percentage of the CBG-bound [3H]cortisol at different times of DCC exposure in relation to the extrapolated CBG-bound [3H]cortisol at zero time of DCC exposure (right panel). Linear regression was used to fit the data, and the r values for the [3H]cortisol binding data were > 0.961.

FIGURE 8. Dissociation of [3H]cortisol from untreated and elastase-cleaved T342P human CBG. A, purified T342P CBG was subjected to elastase cleavage for 5, 15, 30, 60 min at 37 °C, and then analyzed by Western blotting. B, aliquots of the T342P CBG variant before and after elastase-treatment for different times were subjected to a [3H]cortisol-binding assay; the results are presented as the amount of CBG-bound [3H]cortisol after elastase cleavage as a percentage of that obtained without elastase treatment. C, assay to monitor the dissociation of [3H]cortisol from untreated versus elastase-treated T342P CBG. The dextran-coated charcoal (DCC) was added to [3H]cortisol-saturated T342P CBG (untreated or elastase-digested for 15 min or 1 h) at 0 °C, and the samples were incubated for 5, 10, 15 or 20 min at 0 °C before centrifugation to separate CBG-bound from free [3H]cortisol. Aliquots of the corresponding samples were also run on a Western blot to confirm the cleavage by elastase, as illustrated in Figure 8A. The results are presented as CBG-bound [3H]cortisol in cpm (left panel) or the percentage of the CBG-bound [3H]cortisol at different times of DCC exposure in relation to the extrapolated a CBG-bound [3H]cortisol at zero time of DCC exposure (right panel). Linear regression was used to fit the data, and the r values for the [3H]cortisol binding data were > 0.981.

FIGURE 9. Demonstration that human CBG variants (E334A, V336R, G340S, T342P) have higher cortisol-binding affinities than wild-type (WT) human CBG. A, assay to monitor the dissociation of [3H]cortisol from wild-type (WT) and variant (E334A, V336R, G340S, T342P) human CBGs. The dextran-coated charcoal (DCC) was added to the [3H]cortisol-saturated CBGs at 0 °C, and the samples were incubated for 5, 10, 15 or 20 min at 0 °C before centrifugation. The results are presented as the percentage of the CBG-bound [3H]cortisol at different times of DCC exposure in relation to the extrapolated CBG-bound [3H]cortisol at zero time of DCC exposure (right panel). Linear regression was used to fit the data, and the r values for the [3H]cortisol binding data were > 0.984. B, Scatchard analysis of the binding of [3H]cortisol to wild-type (WT) and variant human CBGs (see text for equilibrium binding constant (Kd) values of wild-type and variant human CBGs).

FIGURE 10. Model explaining the increased steroid-binding affinity of some CBG variants with amino acid substitutions in the RCL. We propose that in wild-type CBG, the RCL toggles between two conformational states: (A) a conformation in which the intact RCL is partially inserted between β-strands s3A and s5A of β-sheet A, and (B) a conformation in which the RCL is fully expelled from β-sheet A. In the rat CBG crystal structure, the RCL is fully expelled when the steroid-binding site is occupied (13), and the present data indicate that amino acid substitutions would block RCL insertion (e.g. CBG V336R) result in increased steroid-binding affinity. Thus, if in the absence of steroid, conformation A is the thermodynamically preferred conformation for wild-type CBG, the free energy released upon steroid-binding should be diminished by the amount of energy required to shift the equilibrium towards conformation B. It would then follow that when the partial insertion of the RCL loop is impaired, as in CBG V336R, the steroid-free conformation (C) would resemble the steroid-bound conformation (B) with respect to the positioning of the RCL loop, and hence no energy would be required to shift conformations. As a result, the steroid-binding affinity of variants, such as V336R, would be constitutively increased when compared to wild-type CBG. In the case of V336R, our conclusion that an arginine residue in this position completely blocks RCL insertion is supported by examples of this in other serpin structures (28-30). However, a lack of partial RCL insertion in the native structures of other variants (E334A, G340S, T342P) may involve other most subtle mechanistic explanations.
<table>
<thead>
<tr>
<th>Mutation</th>
<th>Position</th>
<th>Primers (5'-3')*</th>
</tr>
</thead>
</table>
| E334del     | P16      | Forward: GCTGCAACTCAATGAGGGTGTTGGACACACAGCTGG  
Reverse: CCAGCTGTGTCACACCCTTCATTTGAGGTCAGC |
| E334A       | P16      | Forward: GCTGCAACTCAATGAGGCCGGTGGTGACACACAGCTGG  
Reverse: GCTGTGTCACACCCTTCATTTGAGGTCAGC |
| G335P       | P15      | Forward: GCAACTCAATGAGGACACAGCTGGTGACACACAGCTGG  
Reverse: GCCAGCTGTGTCACACAGGCTCCTCTATGAGGTCAGC |
| E334G+G335N | P16+P15  | Forward: GCTGCAACTCAATGAGGGGAATGGTGACACACAGCTGG  
Reverse: GCCAGCTGTGTCACATTTGCCCTCATTGAGGTCAGC |
| V336R       | P14      | Forward: CTCAATGAGGAGGCTGGACACAGCTGGCTGGCTCC  
Reverse: GGAGCCAGCTGTGTCACACCCCTCCTCCTC |
| T338P+A339N | P12+P11  | Forward: GAGGAGGATGGTGACACCCAAATGGCTCCACTGGGTC  
Reverse: GACCCCCAGTGAGCACATTTGGGTCACACCCTCCTC |
| T338P       | P12      | Forward: GAGGAGGATGGTGACACCCACGCTGGCTCCACGTGG  
Reverse: CCAGTGGAGCCAGCTGGTGACACCCCTCCTC |
| G340P       | P10      | Forward: GGTGTGGACACAGCTCCCTCCACTGGGTCACCC  
Reverse: GGTGACACCCAGTGAGGAGCTGGTGACACCC |
| G340S       | P10      | Forward: GGTGTGGACACAGCTCCCTCCACTGGGTCACC  
Reverse: GGTGACACCCAGTGAGGAGCTGGTGACACCC |
| T342P       | P8       | Forward: GGACACAGCTGGCTCCCTGGGTCACCCATAAACCC  
Reverse: GGTGTAGGGTTGACACCCAGGAGCCAGCTGGTG |
**Figure 1**

**A**

<table>
<thead>
<tr>
<th>Species</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>HumanCBG</td>
<td>LKSSLKVLQAVLNEEGVDTAGST--GGLNLTSKPIILRFQEFIMIFDFTWSSLFLARVVPN</td>
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<tr>
<td>RatCBG</td>
<td>LTLT-MVHKAMLQDEGNVLNPSTN--GAPLHLRSEPLDIIKFKNFLILLFDKFTWSSLFLMSQVVNPA</td>
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<td>LTLT-VLHKAMLQDEGNVPLAAATN--GPPVHLPSSEFTLKYNRPFIALFDTWSSLMLMSQVVMNP</td>
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<tr>
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<td>LVSKVLKAVLQDEHGVEVAGAT--GGLQLTVSEPLTLNFRFPILIFDDFTWSSLFLGKVVIPA</td>
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<tr>
<td>PigCBG</td>
<td>PKMSKVLQAVLQDEHGGWEAAGAPTTRGRSLHAAPKFPVTQVFHNRFPFLMTDFFTWSSLFLGKIVNL</td>
</tr>
<tr>
<td>SheepCBG</td>
<td>PKLSKVHLQAVLQDEHGVEVAAGAPTTRGSVLRTFNRFPIMIFDDFTWSSLFLGKVVPN</td>
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<tr>
<td>HumanAAT</td>
<td>LKLSKAVLQAVLQDEHGGWEAAGAPTTRGSVLPFPVTQVFHNRFPFLMTDFFTWSSLFLGKIVNP</td>
</tr>
</tbody>
</table>

**B**

- **P16**: E334 del
- **P16**: E334A
- **P16+P15**: E334G+G335N
- **P15**: G335P
- **P14**: V336R
- **P12**: T338P
- **P12+P11**: T338P+A339N
- **P10**: G340P
- **P10**: G340S
- **P8**: T342P
- **P8**: T342N

**Elastase cleavage site**
Figure 2

A

B

Bound [3H]cortisol after elastase cleavage (% untreated values)

WT  E334G+G335N  E334del  E334A  G335P
elastase  -  +  -  +  -  +  -  +  -  +

75 kDa  →

50 kDa  →
Figure 3

A

B

C

D

E
Figure 4

A

B

Bound [3H]cortisol after elastase cleavage (% untreated values)

WT  V336R  T338P+A339N  T338P
Figure 5

A

B

C
Figure 6

A

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>G340P</th>
<th>G340S</th>
<th>T342P</th>
<th>T342N</th>
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<tbody>
<tr>
<td>elastase</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

Bound [3H]cortisol after elastase cleavage (% untreated values)

B

Bound [3H]cortisol after elastase cleavage (% untreated values)
Figure 7

A

B

C
Figure 8

A

Elastase cleavage (min)  
0 5 15 30 60

T342P

75 kDa

50 kDa

B

Bound \[^{3}H\text{cortisol (%)}

0 5 15 60

Time of Elastase Cleavage (min)

C

Bound \[^{3}H\text{cortisol (cpm)}

0 5 10 15 20

Time of DCC exposure (min)

T342P

T342P cleavage 15 min

T342P cleavage 1 h

Linear fit of T342P

Linear fit of T342P cleavage 15 min

Linear fit of T342P cleavage 1 h
Figure 9

A

![Graph A]

B

![Graph B]
Figure 10
Residues in the human corticosteroid-binding globulin reactive center loop that influence steroid binding before and after elastase cleavage
Hai-Yan Lin, Caroline Underhill, Bernd R. Gardill, Yves A. Muller and Geoffrey L. Hammond

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