Journal of Steroid Biochemistry & Molecular Biology 120 (2010) 30–37

Contents lists available at ScienceDirect

Journal of Steroid Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/jsbmb

Effect of mutations of the human serpin protein corticosteroid-binding globulin on cortisol-binding, thermal and protease sensitivity

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ARTICLE INFO

Article history:
Received 26 October 2009
Received in revised form 25 February 2010
Accepted 2 March 2010

Keywords:
Serpin
Corticosteroid
Hormone binding protein
Transcortin
Cortisol
Elastase
Reactive center loop
Heat sensitivity
CBG Lyon

ABSTRACT

Corticosteroid-binding globulin (CBG, transcortin) belongs to the serpin family of serine protease inhibitors (SERPINA6) and is mainly secreted by the liver. The negative acute phase protein CBG regulates free cortisol levels in the blood and distributes cortisol to its target tissues. So far no CBG serpin partner protease has been identified. However, its cleavage by human neutrophil elastase destroys ligand binding capacity and supposedly liberates cortisol at sites of inflammation. Here we report on the recombinant expression and secretion of human wild-type CBG and several novel mutants by human 293-EBNA cells. Functional characterization of wild-type and mutant CBG revealed distinct differences in ligand binding sensitivity to heat or elastase. Certain mutants are almost devoid of cortisol binding activity (Q232R and CBG Lyon), some display higher sensitivity for heat inactivation (G335V, Q232R and CBG Lyon) or for elastase cleavage (G335V). CBG mutant T342A is more resistant to elastase cleavage. Our data support the validity of the serpin structural concept. The expression system used provides functionally active human recombinant transcortin for further functional characterization of wild-type and human CBG mutant variants, which have been associated with altered serum free cortisol levels or pathophysiological constellations such as increased body weight, fatigue or hypotension.

1. Introduction

Corticosteroid-binding globulin (CBG, transcortin, SerpinA6, OMIM +122500) is a 55 kDa plasma glycoprotein preferentially binding corticosteroids. Serum CBG is secreted by the liver and its main function is the distribution of plasma glucocorticoids to their target tissues and cells. CBG controls the bioavailability of corticosteroids in the blood by regulating the free cortisol concentration, which normally ranges around 5–10% of total cortisol [1,2].

CBG levels in blood are associated with body mass index, insulin resistance [3], serum levels of interleukin-6 [4] and adiponectin [5] and with proliferation and differentiation of preadipocytes [6]. Furthermore, a decreased CBG level during acute pancreatitis has been suggested as an early predictor of a later infected pancreatic necrosis [7]. Naturally occurring mutations in the gene encoding CBG have been identified [8–11] that result in CBG variants with reduced cortisol binding activity or in non-functional CBG due to premature termination of translation [12]. Some of these mutations are associated with fatigue and hypotension [4,10]. Decreased hepatic expression and secretion of CBG has been reported to transiently increase circulating free corticosterone levels in mice resulting in thymic involution [13]. Mice genetically deficient in CBG show a remarkably mild phenotype and normal development, but exhibit increased activity of the pituitary adrenal axis and enhanced susceptibility to septic shock, indicating an inappropriate response to elevated corticosteroids [14] (see Gagliardi et al. [15] and Moisan [16] for recent reviews).

CBG is a member of the serine protease inhibitor (Serpin) superfamily. Upon interaction with and cleavage by plasma proteases, serpins initiate irreversible conformational changes from a stressed (S) to a relaxed (R) conformation, which results in the inhibition of both the serpin and the protease function. The major structural change of the serpin induced by protease cleavage leads to a movement of the reactive center loop (RCL) from its exposed position to an integrated beta strand in beta-sheet A [17], furthermore important conformational changes of helix D were shown for CBG [18,19] (see Fig. 1). In active serpins a covalent complex between protease and serpin is formed. Cleavage of the serpins increases their stability against thermal and chemical denaturation [20,21]. CBG is considered as inactive serpin as it has no known protease inhibitory function, but is acting as a substrate for human neutrophil elastase (HNE) [22]. CBG is cleaved by HNE between a valine

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doi:10.1016/j.jsbmb.2010.03.014

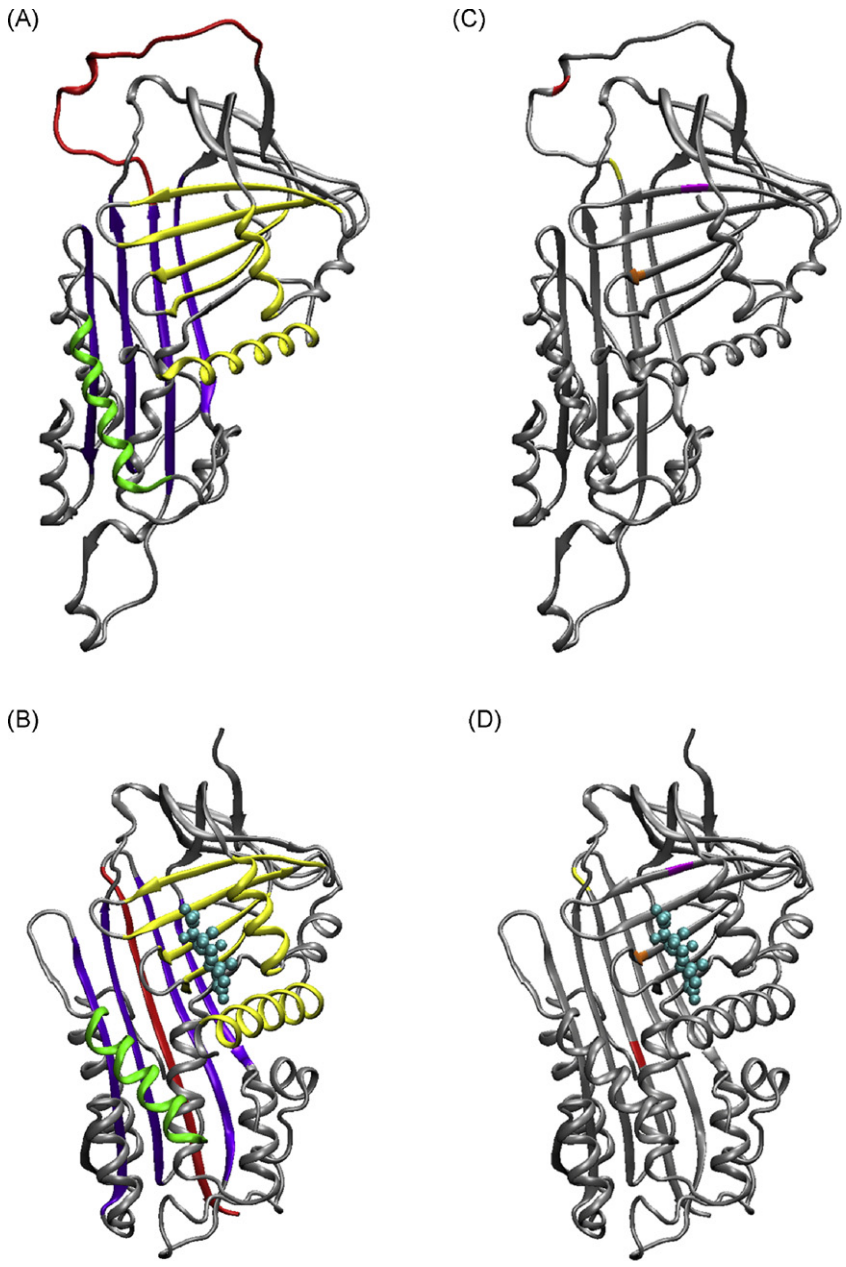

Fig. 1. Structure models of CBG. On top (A and C) the rat CBG in S-state conformation (modification of Protein Data Bank code 2v95) at bottom (B and D) the human cleaved CBG:AAT chimera structure with cortisol (the ligand is cyan colored) in the R-state conformation (Protein Data Bank code 2VDY). (A and B) Special structures. RCL, red; beta-sheet A strands s2A, s3A, s5A, s6A, violet; helixD, green; binding pocket (beta-sheet B strands s2B, s3B, s4B, s5B, helixA and helixH), yellow. (C and D) Localization of the investigated amino acid substitutions. The positions of the amino acids substituted in human CBG in our study and of their homologues in rat CBG, respectively, are color marked with yellow (G335) and red (T342) (both residues located in the RCL), respectively with magenta (Q232) and orange (D367) (both residues are part of the binding pocket). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article)

and a threonine residue in the RCL which is shown in Fig. 2. The single cut of CBG by HNE causes a strong decrease of the cortisol binding activity [22]. It can be assumed that this represents a mechanism for the local or systemic release of high amounts of free cortisol at sites of inflammation [23]. So the view of CBG representing only an inert transport, storage and distributor protein for corticosteroids is changing to the concept of a protein acting as an important regulator of free local corticosteroid concentrations, e.g. during inflammation. Specific interactions of CBG with proteases other than HNE have not been reported until now.

Thyroxine-binding globulin (TBG) represents another "inactive" serpin, which can also be cleaved by HNE. The importance of the RCL-region for heat and protease sensitivity and the impact of this region on the serpin conformation and the ligand binding properties were shown by Janssen et al. and Grasberger et al. [24,25]. They designed TBG-alpha1-proteinase inhibitor chimeras modified in the loop region. One of them contains a shortened loop (TP_{L1}) and one a prolonged loop (TP_{L2}). The group proposed that TP_{L2} drives the serpin conformation more to the relaxed state, which is characterized by a decreased thermo and/or protease sensitivity but a lower ligand affinity. The opposite is true for TP_{L1}, i.e., the shorter loop chimera was less stable but displayed higher affinity. For both mutants a cleavage by HNE was shown.

Dey and Roychowdhury proposed a structure model of human CBG and suggested amino acids involved in ligand binding (aa Ser219, Gln232, Asn234, Asn238, Ile263, Ser267, Phe369, Trp371) based on a sequence homology with alpha1-antitrypsin (A1AT) and alpha1-antichymotrypsin (AACT), two active serpin family mem-

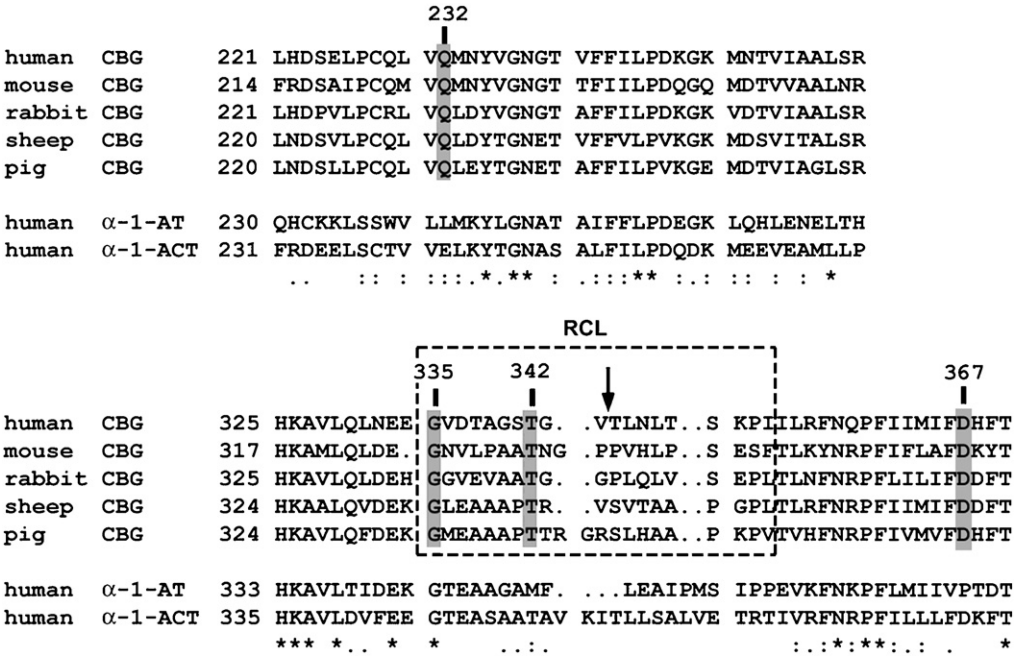

Fig. 2. Multiple sequence alignment among CBG sequences from different species and α-1-anti-trypsin (α-1-AT) and α-1-antichymotrypsin (α-1-ACT). The gray shaded boxes indicate the residues of interest. The arrow indicates the site of cleavage of CBG by neutrophil elastase. The less conserved C-terminal reactive center loop (RCL) of CBG is highlighted by the box. "*" means that the residues in that column are identical in all sequences in the alignment, ":" means that conserved substitutions have been observed, "." means that semi-conserved substitutions are observed.

bers that share a remarkable sequence homology with CBG of 52.2 and 55.5%, respectively [26]. Their model has been confirmed and more detailed information on the ligand binding site, the dynamics of RCL during protease cleavage and conformational changes involved have been reported by Klieber et al. [19] and Zhou et al. [18] for the crystal structures of rat and human CBG. In contrast to human TBG rat CBG displays an active serpin conformation similar to thrombin inhibitory serpins [19]. Lin et al. analyzed in detail the effects of amino acid mutations of the hCBG RCL on ligand binding in the context of HNE cleavage [27].

In this paper we report on the recombinant *in vitro* expression of functional human CBG and mutants thereof. Based on the structural serpin concept we characterized two designed amino acid mutants of the reactive center loop region, one further mutation located in the potential ligand binding pocket and in addition the naturally occurring CBG Lyon-mutation, also positioned in the binding pocket. We functionally tested and experimentally confirmed some predictions of the models and structures proposed by Dey and Roychowdhury [26], Klieber et al. [19] Zhou et al. [18] and Lin et al. [27] with respect to ligand binding and role of the serpin RCL. Here we present as new information comparative data with respect to protease sensitivity and thermolability of these CBG mutants. We also provide this information for two ligand binding pocket mutants (CBG Lyon and Q232R) which show low ligand affinity, one of them found in humans with a clinical phenotype of fatigue and hypotension.

2. Materials and methods

2.1. Generation of CBG, His-CBG and His-CBG mutants

Human cDNA was generated by SuperScript II reverse transcriptase (Invitrogen) from human liver (BD Biosciences) and HepG2 RNA, respectively. The complete reading frame of human CBG was amplified by PCR, cloned, and confirmed by sequencing using gene bank accession number NM_001756 as source (list of all used primers pairs in Table 1). Different mutants of CBG were generated by site directed mutagenesis using overlapping PCR-fragments. Sequences were cloned into the pGEM-Teasy-vector (Promega) and verified by sequence. The following mutations were generated: His-Q232R, His-G335V, His-T342A and His-D367N, whereas mutant His-Q232R was obtained by a spontaneous mutation during PCR-amplification (numbering with respect to mature CBG, i.e., without signal sequence). For functional analyses we employed two different expression vectors. Wild-type CBG cDNA including the signal sequence was subcloned into pCEP-Pu [28,29] to obtain CBG without a tag. Alternatively, wild-type CBG and the different CBG mutants without CBG signal sequence were subcloned into the pCEP-Pu-SP-his-myc-fX vector [28,29], which provides an N-terminal human BM-40 signal sequence and a His_6-tag.

2.2. Recombinant expression and purification

Human embryonic kidney cells (293-EBNA cells, Invitrogen) were transfected with the different CBG constructs using lipofectamine (Invitrogen). The transfected cells were grown in DMEM (Biochrom) supplemented with 10% fetal calf serum (FCS, Biochrom), 2 mM L-glutamine (Gibco), 250 μg/ml G418 (Calbiochem) and 1 μg/ml puromycin (Sigma). At 80% confluence, medium was exchanged for FCS- and phenolred-free DMEM (Biochrom), 2 mM L-glutamine, 250 μg/ml G418, 0.5 μg/ml puromycin and 50 nM cortisol (Sigma). After 3–4 days of incubation, the medium was collected, centrifuged and filtrated (0.45 μm filter). The resulting supernatants were applied to Ultrafree-15 units (Millipore) to concentrate the secreted proteins.

For purification of CBG without His-tag the concentrated supernatant was buffered in PBS containing 10% glycerol, and was then applied to a cortisol-affinity column (Cortisol-CMO (Sigma) was covalently coupled to EAH-Sepharose (GE Healthcare) by EDC (Pierce)). After removal of the flow-through, the column was washed several times with PBS containing 10% glycerol. Bound CBG was eluted by addition of 50 μM cortisol to the wash-buffer (PBS, 10% glycerol).

Table 1
Primer sequences used for PCR-amplification of human CBG, His-CBG and human His-CBG mutants. Different mutants of CBG were designed by site directed mutagenesis using overlapping PCR-fragments (fragment 1a and 1b) resulting in fragment 1. The complete sequences were results of overlapping PCR-fragments 2 and 1.

	Primer pairs	Sequence
ppCBG		
Complete sequence	s-KpnI-pphCBG	5′-GGTGGTACCATGCCACTCCTCCTGTACAC-3′
	as-XhoI-hCBG	5′-CTCCTCGAGTTACACTGGGTTCATAACCCTC-3′
mpCBG		
Complete sequence	s-NheI-mphCBG	5′-GCTGCTAGCCATGGATCCTAACGCTGC-3′
	as-XhoI-hCBG	5′-CTCCTCGAGTTACACTGGGTTCATAACCCTC-3′
mpCBG_G335V		
Fragment 1b	ms-SacI-hCBG	5′-GCAGAGCTCCCCTGCCAGC-3′
	as-Gly335Val-hCBG	5′-GTGTCCACAACCTCCTCATTG-3′
Fragment 1b	s-Gly335Val-hCBG	5′-CAATGAGGAGGTTGTGGACAC-3′
	as-XhoI-hCBG	5′-CTCCTCGAGTTACACTGGGTTCATAACCCTC-3′
mpCBG_T342A		
Fragment 1a	ms-SacI-hCBG	5′-GCAGAGCTCCCCTGCCAGC-3′
	as-Thr342Ala-hCBG	5′-GTGACCCCAGCGGAGCCAG-3′
Fragment 1b	s-Thr342Ala-hCBG	5′-CTGGCTCCGCTGGGGTCAC-3′
	as-XhoI-hCBG	5′-CTCCTCGAGTTACACTGGGTTCATAACCCTC-3′
mpCBG_D367N		
Fragment 1a	ms-SacI-hCBG	5′-GCAGAGCTCCCCTGCCAGC-3′
	as-Asp367Asn-hCBG	5′-GTGGTTGAAGATCATGATGATG-3′
Fragment 1b	s-Asp367Asn-hCBG	5′-CATCATCATGATCTTCAACCAC-3′
	as-XhoI-hCBG	5′-CTCCTCGAGTTACACTGGGTTCATAACCCTC-3′
All mutants[a]		
Fragment 1	ms-SacI-hCBG	5′-GCAGAGCTCCCCTGCCAGC-3′
	as-XhoI-hCBG	5′-CTCCTCGAGTTACACTGGGTTCATAACCCTC-3′
Fragment 2	s-NheI-mphCBG	5′-GCTGCTAGCCATGGATCCTAACGCTGC-3′
	as-SacI-hCBG	5′-GCTGGCAGGGGAGCTCTGC
Complete sequence	s-NheI-mphCBG	5′-GCTGCTAGCCATGGATCCTAACGCTGC-3′
(Fragment 1 + 2)	as-XhoI-hCBG	5′-CTCCTCGAGTTACACTGGGTTCATAACCCTC-3′

"s" or "ms", sense primer; "as", anti sense primer.
[a] Without mutant His-Q232R.

For purification of His-CBG and the His-tagged mutants ("His-" denotes the N-terminal His_6-tag) the concentrates were buffered in PBS, 10% glycerol, 1 μM cortisol, and applied onto a chelating Sepharose (GE Healthcare) pretreated with nickel chloride. After intensive washings, the His-tagged CBG was eluted by adding increasing amounts of imidazol to the binding buffer. The main fraction of His-CBG, which elutes at 100 mM imidazol, was equilibrated with 10 mM phosphate buffer pH 6.8, 100 mM NaCl and applied to a Hydroxyapatite Typ I 40 μm (BioRad) column. Under these conditions CBG does not bind to the column and is found in the flow-through. All purified proteins were stored in 10 mM Hepes pH 7.4, 150 mM NaCl, 0.005% Surfactant P20 (GE Healthcare), 10% glycerol at −70 °C until analysis.

2.3. Corticosteroid-binding assay

Purified CBG and His-CBG mutants were stripped from endogenous steroids by incubation with dextran-coated charcoal (DCC) treatment in PBS containing 1 mg/ml collagen for 30 min at 25 °C. DCC was precipitated by centrifugation and the resulting supernatants were used for the corticosteroid-binding assay. Binding of steroid hormones to proteins was carried out according to Hammond and Lahteenmaki [30]. The samples were supplemented with increasing amounts (Scatchard analysis) or an excess (normal binding assay) of [^{3}H]cortisol ([^{3}H]hydrocortisone, Perkin-Elmer, 70–80 Ci/mmol). A first incubation for 20 min at 37 °C was followed by a second incubation for 15 min on ice. The amount of CBG-bound [^{3}H]cortisol was measured after 10 min DCC treatment on ice and centrifugation at 4 °C and counting of the resulting supernatant in a liquid scintillation counter (Perkin-Elmer). To determine the technical background for Scatchard analysis, the steroid-binding assays were additionally performed without the steroid-binding protein. The specific background was measured by adding a 200-fold excess of unlabeled cortisol to the reaction mixture.

2.4. CBG structure model presentation

For the presentation of the localization of our amino acid substitutions in CBG structure models we used the program VMD [31] and the structure data of the cleaved human CBG:AAT chimera structure with ligand (Protein Data Bank code 2VDY [18]) and a SWISS-Model modified form [32,33] of rat CBG (Protein Data Bank code 2v95 [19]).

2.5. Temperature sensitivity of CBG variants

Steroid-free corticosteroid-binding proteins (CBG and different His-CBG mutants) were incubated at 50 or 55 °C for 0, 10, 20 and 40 min. One part of the resulting samples was supplemented with native sample buffer and separated by native polyacrylamide gel electrophoresis. CBG, His-CBG and His-mutants were visualized after semidry western blotting by immunostaining using a rabbit anti-human CBG antibody (Dako) or a mouse anti-Penta-

His-tag antibody (Qiagen). The cortisol binding of CBG, His-CBG and several His-CBG mutants after heat treatment were tested by the [^{3}H]corticosteroid-binding assay described above.

2.6. Human neutrophil elastase digestion assay

0.25 μg of corticosteroid-binding protein (CBG or His-CBG mutants) was pre-incubated with DCC in PBS containing 1 mg/ml collagen for 30 min at 25 °C. After removal of the DCC by centrifugation, the supernatant was incubated with 0.5 μg human neutrophil elastase (Calbiochem) in PBS for 10 min at 37 °C. The digestion was stopped by trichloracetic acid (TCA) precipitation (final TCA concentration, 20%). The resulting CBG fragments were separated by denaturing SDS-PAGE (10% separation gel) and visualized after semidry western blotting by immunostaining using an anti-CBG antibody as described above. The corticosteroid-binding of CBG and His-CBG mutants were measured by the [^{3}H]corticosteroid-binding assay. After pre-incubation with DCC, the resulting supernatants were mixed with an excess of [^{3}H]cortisol in PBS and were incubated for 20 min at 37 °C. After 5 min at 0 °C, the samples were digested with 0.5 μg human neutrophil elastase for 5 or 10 min at 37 °C. The subsequent DCC extractions were performed under standard conditions to determine the steroid-binding (see above).

2.7. Statistics

Data represent independent experiments performed at least three times and mean values ± standard deviation are presented.

3. Results

We were interested to create mutants of the RCL region (Figs. 1 and 2) that plays a central role in the protease dependent release of CBG-bound cortisol. Therefore, we chose two amino acids in the reactive loop, G335 and T342 for substitutions. Both G335 and T342 are highly conserved between different species; moreover G335 is also conserved in other members of the serpin family such as alpha1-antitrypsin or alpha1-antichymotrypsin (Fig. 2). In our experiments G335 was substituted by valine (His-G335V) and T342 was substituted by alanine residue (His-T342A). Additionally, we generated the naturally occurring CBG Lyon-mutation (His-D367N). Furthermore, several groups [18,19,26,34] specified Q232 as important for corticosteroid-binding. Q232 is highly conserved between different species (Fig. 2) and it seems to be important for the conformational stability in a subdomain of the binding region. We generated a His-tag mutant, with glutamine replaced by arginine (His-Q232R). The localization of the substitutions in the relaxed (R) conformation as well as in the stressed (S) conformation is illustrated in Fig. 1.

In order to characterize the cortisol binding activity of the generated mutants, we measured the binding of tritium-labeled cortisol by charcoal–dextran-assay. CBG, His-CBG and the mutants His-G335V and His-T342A were capable to specifically bind cortisol within the same range (Fig. 3). Obviously, the substitutions in the RCL did not influence considerably cortisol binding. In contrast, both mutants with substitutions in the predicted cortisol binding center, mutant His-Q232R and His-D367N (the latter corresponding to the naturally occurring CBG Lyon mutant), show remarkably weaker cortisol binding compared to wild-type CBG.

The RCL mutants His-G335V and His-T342A were analyzed in more detail. Scatchard plots of cortisol binding of CBG, His-CBG, His-G335V and His-T342A were established (Fig. 4). Wild-type CBG (K_d = 31 nM) and His-T342A (K_d = 32 nM) show the highest binding of all proteins tested and nearly the same K_d values (delineated from Scatchard plot slopes), which are lower than those for His-CBG or His-G335V. The mutant His-G335V has a lower K_d (K_d = 14 nM)

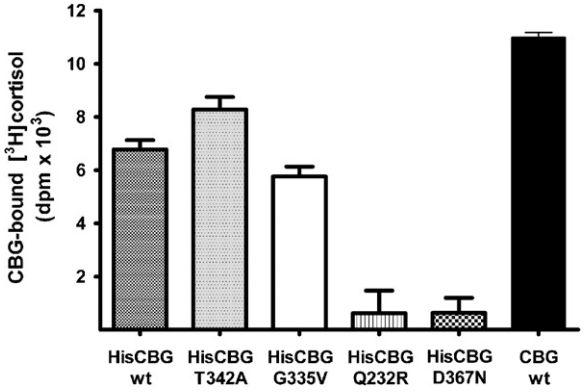

Fig. 3. Different steroid-binding of CBG and His-CBG mutants. [^{3}H]cortisol was added to different CBG variants. The amount of protein-bound steroid was determined after treatment with DCC for 10 min at 0 °C. Bars shown are representative of three independent experiments. Values are the means of duplicates.

resulting in a higher binding affinity for cortisol in comparison to wild-type His-CBG (K_d = 23 nM).

Mutations of conserved residues in the RCL and of the proposed ligand binding site of CBG were also analyzed with respect to their thermolability. Therefore, CBG, His-CBG and the mutants were incubated in a pilot experiment at different temperatures (not shown). Pre-incubations at 50 or 55 °C over a time period of 40 min resulted in detectable differences in functional protein properties. Subsequently, the proteins were analyzed in binding assays and on native acrylamide gels. Fig. 5A illustrates that proteins not exposed to elevated temperature show only one strong immunoreactive band with the highest mobility. In all cases heat treatment decreased intensity of the major band with time while higher molecular mass immunoreactive bands appear. The total intensity of immunodetectable proteins decreased with prolonged heat treatment. CBG and His-CBG showed similar properties in the binding assay (50 and 55 °C, Fig. 5B) and in native gels (55 °C, Fig. 5A). For His-T342A no significant difference in comparison to wild-type could be shown. In contrast, His-G335V, His-Q232R and His-D367N were more sensitive to higher temperatures. Western Blot analyses revealed a faster disappearance of the band with the highest mobility for these three mutants compared to wild-type His-CBG after native gel electrophoresis. No thermal ligand binding profile could be obtained for mutant His-Q232R and His-D367N due to their very low binding activity.

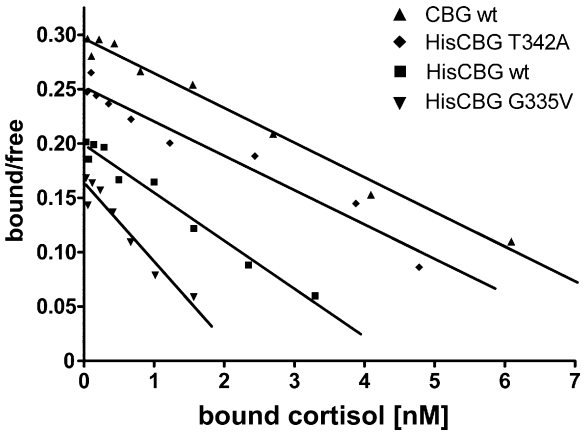

Fig. 4. Scatchard analysis of CBG and His-CBG mutants. The cortisol binding properties of His-CBG (■), CBG (▲), His-G335V (▼) and His-T342 (♦) were analyzed by Scatchard plot. Plots shown are representative of three independent experiments.

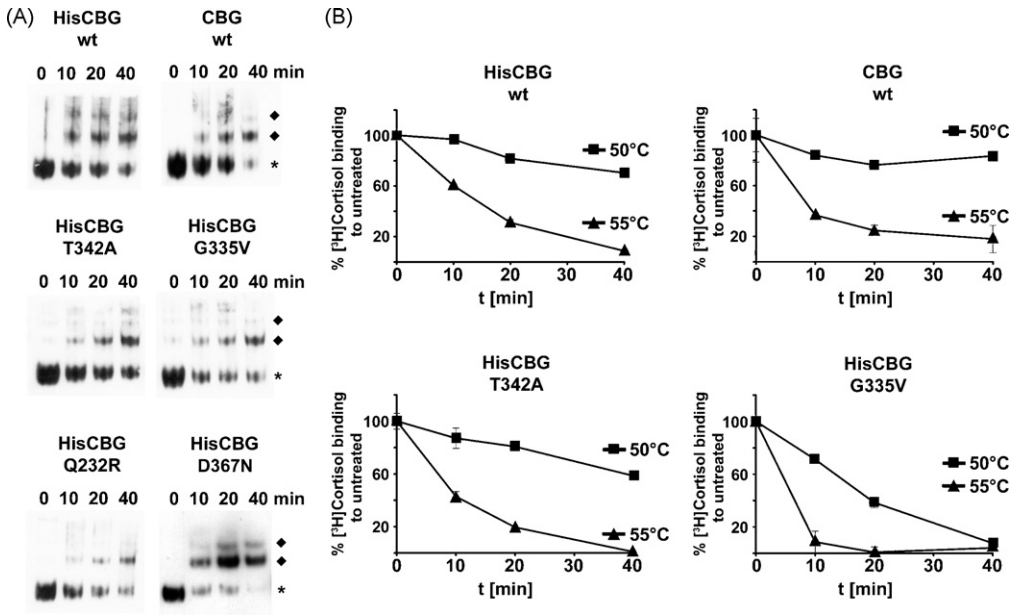

Fig. 5. Thermolability of purified CBG and His-CBG mutants at 50 and 55 °C. (A) CBG and different His-CBG mutants were incubated at 55 °C for 0, 10, 20 and 40 min. Non-denaturating sample buffer was added after heat treatment and the samples were separated by non-denaturating PAGE. CBG and His-CBG were visualised by Western blotting and immunostaining using an anti-His-tag antibody (His-CBG) or an anti-CBG antibody (CBG). The resulting immunoreactive band with the highest mobility is marked by an asterisk, lower mobility bands were marked by a rhomb. (B) The cortisol binding of CBG, His-CBG, His-G335V and His-T342 after heat treatment at 50 °C (■) or 55 °C (▲) for 0, 10, 20 and 40 min were measured using the [^{3}H]cortisol binding assay. Mock-treated samples were taken as 100% cortisol binding, respectively. Graphs shown are representative of three independent experiments. Values are the means of duplicates. The binding-inactive mutants His-Q335V and His-D367N were omitted.

HNE is used as a model protease interacting with the serpin loop of CBG. In order to get information on the impact of these novel CBG mutants on serpin loop–protease interaction we incubated our mutants with HNE. The wild-type and the mutant proteins were all cleaved by HNE, albeit to different degrees (Fig. 6A). His-T342A turned out to be more resistant to HNE cleavage than wild-type His-CBG. Over time the binding activity of this mutant did not decrease as fast as the binding activity of wild-type His-CBG, indicating that His-T342A is more resistant to HNE than wild-type His-CBG. Mutant His-G335V has a higher sensitivity for HNE cleavage compared to all the other proteins tested (Fig. 6B).

4. Discussion

In this study we characterized binding properties, heat and protease sensitivity of recombinantly produced and purified human CBG, His-CBG and selected mutants. We have chosen a homologous human kidney cell model for recombinant expression of human CBG variants in order to achieve a near-natural state of glycosylation [35,36]. We observed a difference between CBG and His-CBG in cortisol binding which can have different reasons. Firstly, the N-terminal His-tag is located close to the proposed binding site in the globular CBG structure and might influence the access of steroids to their binding site. Secondly, two different purification methods were used for CBG versus His-CBG. In the case of CBG only active cortisol binding protein was purified by the affinity chromatography procedure. In contrast, for His-CBG a co-purification of binding-inactive variants could not be excluded because the purification occurred by the affinity of their His-tag for metal ions. This purification was used to enable comparison between His-CBG and the His-CBG-mutants without interference of the affinity purification step.

According to the structure models of CBG (Fig. 1) and our alignment of the sequence of different species (Fig. 2), specific mutations of the two conserved amino acids in the RCL of CBG should induce changes in the binding activity and perhaps in sensitivity against heat and protease, too. Therefore, we supposed that our loop mutants influence CBG conformation, but in which direction – more stressed or more relaxed – could not be predicted. We found that both RCL mutants still bind cortisol. From the Scatchard plot data we conclude that the mutant His-G335V has a higher binding affinity than wild-type His-CBG but a lower binding in comparison. This may be due to the lower stability of this mutant as shown by the increased sensitivity towards heat and HNE treatment. The mutant His-T342A displays a lower binding affinity than the wild-type His-CBG protein, but exhibits a higher maximal binding capacity. While this work was in progress Lin et al. [27] published a report about different RCL mutations. They found a lower K_d value for their T342P mutant and nearly the same K_d value for the mutant T342N in comparison to wild-type CBG. Therefore different substitutions in the RCL can cause different exposition/insertion states of this loop and, in this way, different affinities. Thus it is conceivable that our His-T342A mutant slightly increase the K_d compared to His-CBG.

The mutant His-G335V is noticeable more heat labile than wild-type CBG, His-CBG and also the His-T342A mutant. The higher cortisol binding affinity of the heat sensitive variant His-G335V is analog to one TBG mutant. The short loop “stressed” TBG mutant displayed increased binding to its ligand thyroxine, but was more heat labile than the wild-type TBG. In contrast, the prolonged loop “relaxed” TBG mutant had an impaired thyroxine-binding, but was much less heat labile than the wild-type protein [21]. Thus, we can assume that the conformation of the His-G335V mutant tends more to the stressed version, which is characterized by higher temperature sensitivity. The generally observed decreased intensity of immunoreactivity of heat-treated CBG isoforms (Fig. 5A) can have several reasons. In nondenaturing PAGE CBG oligomers do not enter the native gels easily and transfer of heat-induced oligomers is usually difficult during blotting. Furthermore, immunoreactive epitopes may be masked during the process of oligomerisation.

Cleavage of the CBG mutant His-G335V by HNE is also accelerated compared to wild-type His-CBG indicating structural differences. Lin et al. reported maintenance of binding activity after cleavage of their G335P mutant [27]. They assume that the loop does not (fully) insert into the central β-sheet in such mutations that have slightly decreased or full cortisol binding after HNE cleav-

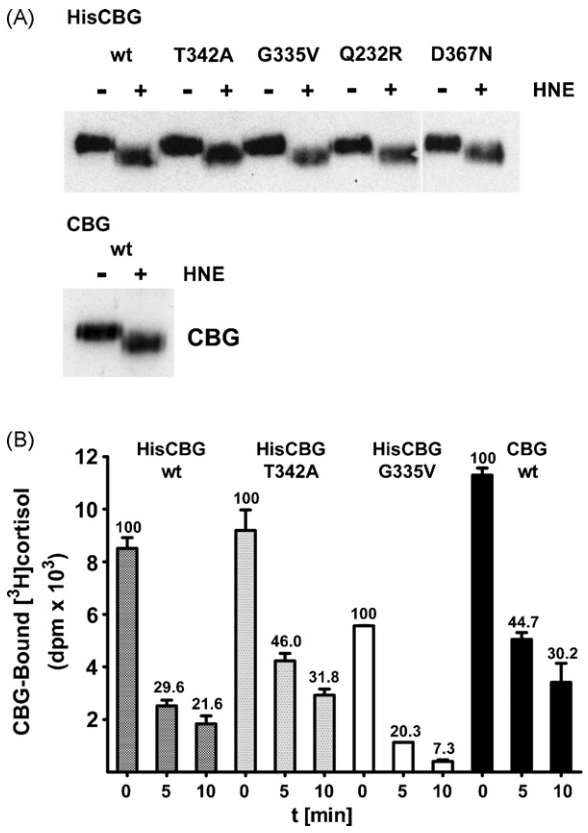

Fig. 6. Effects of neutrophil elastase on cortisol binding by CBG and different His-CBG mutants. (A) The different CBG variants were incubated in the presence or absence of neutrophil elastase for 10 min at 37 °C. The resulting digestion fragments were separated by SDS-PAGE (10%) and visualised by Western blotting and immunostaining using anti-CBG antibody. (B) Equal amounts of the different CBG variants were pre-incubated with [^{3}H]cortisol and then digested with neutrophil elastase for 0, 5 or 10 min at 37 °C. The amount of CBG-bound cortisol was measured after 10 min DCC treatment and centrifugation by liquid scintillation counter. Numbers at the top of the bars indicate the cortisol binding activity (in %) in comparison to mock-treated samples. Bars shown are representative of three independent experiments. Values are the means of duplicates. The binding-inactive mutants His-Q335V and His-D367N were omitted.

age [27]. Decreased cortisol binding in our G335V mutant might indicate that a substitution of glycine against valine does not hinder an insertion of the cleaved RCL while proline appears to do so. Although the Western blot signal reveals a complete shift of the signal after 10 min for all tested proteins, indicating complete cleavage, the loss of binding activity during cleavage over the time proceeded significantly slower for the mutant His-T342A than for wild-type His-CBG. Conceivably, the RCL might have undergone a conformational change in the mutant, which is relevant not only for cortisol binding but also for the access of HNE to its cleavage site. His-T342A in comparison to His-CBG was more stable against HNE but had a lower binding affinity. These observations suggest that the His-G335V mutant is a CBG variant, which can better bind cortisol and could faster supply cortisol to inflammatory sites than wild-type CBG. In contrast, the His-T342A mutant might allow a slower release of cortisol to these sites.

In the case of the mutant His-Q232R we intended to test the proposed structural models [18,19,26,27] and especially to evaluate the participation of this amino acid residue in ligand binding. We observed a drastically reduced binding activity of this mutant using our standard assay and also by employing modified [^{3}H]cortisol binding tests. Both the increased size and the more positive charge of the arginine side chain can lead to decreased binding of cortisol. This provides experimental evidence for the assumption of Edgar and Stein [34], Dey and Roychowdhury [26], Klieber et al. [19] and Zhou et al. [18] that this amino acid is important for and involved in ligand binding. The residue Q232, which is conserved in rat (Q224), pig (Q231), and human CBG (Q232) and in human TBG (Q238), has recently been shown to contact the C20 carbonyl oxygen of cortisol via a hydrogen bond in the rat CBG-cortisol crystal structure [19] and in the human cleaved CBG-cortisol structure [18]. Apparently the longer and positively charged 232 arginine residue distorts binding of cortisol to the ligand binding site formed between sheet B, helix H and helix A and which also involves hydrogen bonds between the C21 hydroxy group, Q224 and G259 in rat CBG [19]. However, the His-Q232R mutant exhibited apparently higher heat lability. A change in conformation, a possible reason for loss of stability, has not been expected by theoretical considerations.

Using serum of homozygous or heterozygous CBG Lyon patients and controls, Emptoz-Bonneton et al. and Brunner et al. [10,11] observed gene-dose-dependent CBG serum concentrations and a reduced affinity for cortisol binding. We confirm their observation also *in vitro* with our recombinantly expressed and purified His-D367N human mutant that shows very low binding of cortisol and increased heat lability. In rat CBG the corresponding residues F357, D358 and K359, which are also conserved in human and pig CBG, have been shown to contribute to the cortisol ligand binding site, though K359 does not reach the C17 hydroxy group to form a hydrogen bond [19]. Zhou et al. have shown a participation of the neighboring H368 in cortisol binding of human cleaved CBG by forming a hydrogen bond with it [18]. The replacement of the negatively charged aspartate 367 by the uncharged asparagin residue in the human CBG Lyon mutant will probably disrupt the structure of the 4B β-sheet of the CBG ligand binding site [19] and its interaction with the D-ring, the C17 hydroxy group and the side chain of cortisol or disrupt the participation of this amino acid in inter-residue contacts that might transmit any changes in helix D to the steroid-binding side [19]. Helix D has been proposed as one of the key coupling elements for the positioning of the RCL and the hormone binding to the steroid-binding site [18,19].

Availability of recombinant wild-type CBG and specific mutants is a prerequisite to analyze structural and functional implications derived from the serpin model of CBG in molecular aspects. Here, we have provided experimental evidence for Q232 as a critical residue involved in cortisol ligand binding as suggested and documented [18,19,26]. In addition, some of our RCL mutants of human CBG showed altered thermolability or HNE protease sensitivity, thus supporting the RCL serpin loop model and providing additional functional information. These positive results support our recombinant expression system as suitable approach to test further predictions from homology-based structural models and available crystal structures for functionally relevant features of human CBG and its mutants.

Acknowledgements

We gratefully acknowledge the extensive grant support by the Technologiestiftung Berlin, Zukunftsfonds, EFRE and Bayer Schering Pharma Berlin. We thank Dr. Zida Wu and Prof. Christian Strasburger for the kind donation of CBG antibody and valuable support. Dr. Thomas Willnow kindly provided the expression vector and cell line. Also we want to thank Dr. Lutz Schomburg and Dr. Joachim Weitzel for helpful discussions and critical reading of the manuscript and Jenny Merz for engaged technical assistance.

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