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Corticosteroid-binding globulin reactive centre loop antibodies recognise only the intact natured protein: Elastase cleaved and uncleaved CBG may coexist in circulation

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ABSTRACT

Corticosteroid-binding globulin (CBG) is the principal carrier of cortisol in circulation and is a non-inhibitory member of the serpin family of serine proteinase inhibitors. It possesses an exposed elastase specific site which, when cleaved, allows a conformational change promoting the delivery of cortisol to sites of inflammation. Previously there was no ability to independently distinguish between the uncleaved, stressed, conformer of CBG and total CBG in circulation. Here we raised and characterized monoclonal antibodies generated against a synthetic peptide spanning the elastase cleavage site within the exposed reactive centre loop (RCL) and measured changes in CBG by ELISA following treatment with human neutrophil elastase. The antibodies recognized the synthetic peptide as well as intact CBG and the epitope (STGVTLNL) spanned the elastase cleavage site. Treatment of plasma with elastase resulted in a complete loss of CBG levels determined using these RCL antibodies whereas CBG levels measured with an unrelated CBG monoclonal antibody were unaffected. We also compared plasma levels of CBG measured by RCL antibodies and an unrelated CBG antibody and showed discordance in some samples. This study shows for the first time the ability to measure the intact, stressed conformer of CBG. We report discordance with total CBG in some samples implying the presence of cleaved CBG in circulation. This is an important finding as it has implications for free cortisol which hitherto have been determined from total cortisol and total CBG levels. This antibody could be used for determining the time course of intact CBG in various relevant patient cohorts and for structure/function studies on the biology of human CBG.

1. Introduction

Corticosteroid-binding globulin (CBG) is a circulating glycoprotein that binds up to 90% of plasma cortisol with high affinity which, along with albumin, serves to help regulate the tissue distribution of free cortisol [1]. In addition to its transport role CBG may directly interact with membrane receptors acting as an alternative glucocorticoid delivery system [2] as well as by the localised release of cortisol at sites of inflammation following cleavage of CBG by elastase. It has been recognised for some time that CBG is a non-functional member of the serine protease inhibitor family with an elastase cleavage site between amino acid residues 344–345 [3]. This elastase cleavage site forms part of the exposed reactive centre loop (RCL) comprising amino acid residues 334–351. In intact or uncleaved CBG, cortisol is tightly bound and the protein exists in a "stressed" or S conformational state [4,5]. However when the RCL is cleaved a 4-kDa C-terminal peptide is evident following denaturing electrophoresis [6] and CBG adopts a transition to the irreversible "relaxed" or R conformation where the cleaved reactive centre loop becomes inserted deep within the protein core to form a novel β-strand [7]. Associated with this S → R transition is the prevailing view of a decrease or loss in affinity for cortisol [8] thus allowing its release directly at sites of inflammation although it seems that cleaved CBG may still complex effectively with cortisol [9]. These structural studies have been carried out using recombinant CBG. We therefore sought to investigate changes in native CBG following elastase cleavage by using monoclonal antibodies directed at the RCL of human CBG. We also used these antibodies to directly determine the presence of intact CBG in human plasma and show that both intact and cleaved CBG may be present in circulation.

2. Materials and methods

2.1. Synthetic peptides and conjugation to carrier proteins

A peptide within the RCL region corresponding to amino acid residues 335–351 of human CBG (GVDTAGSTGVTLNLTSK-C) was synthesized and purified with a C-terminal cysteine.

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Another peptide, C-terminal to the elastase cleavage site, corresponding to amino acid residues 345–362 of human CBG (TLNLTSKPIILRFNQPFI) was also synthesized and purified by Mimotopes Pty Ltd., Vic, Australia. These peptides were designated RCL peptide and RCL C-terminal peptide respectively. The RCL peptide was conjugated to both bovine serum albumin (BSA) and bovine thyroglobulin (BTG) via the added C-terminal sulphydryl group following activation of the proteins with the water soluble heterobifunctional cross-linker sulphosuccinimidyl 4-(*N*-malemidomethyl)-cyclohexane-1-carboxylate [10]. The RCL peptide–BSA was used for immunization and RCL peptide–BTG conjugate for antibody screening. For epitope mapping an additional series of 10 mer peptides were synthesized (unpurified) that spanned the elastase cleavage site, each differing incrementally by two amino acids. These sequences are shown in Fig. 4.

2.2. Immunization and cell fusion

Two female BALB/c mice were immunized with 10 μg of RCL peptide–BSA conjugate in complete Freunds adjuvant at 4-week intervals. One week after the 4th injection, spleens were excised and fused with FOX-NY myeloma cells at a ratio of 5:1 as described previously [11].

2.3. Screening of supernatants

Supernatants were screened using 2 parallel methods. Firstly ELISA plates (Falcon 3912 Microtest III; Becton-Dickinson, Oxnard, CA, USA) were coated a few days in advance with either RCL peptide–BTG conjugate in 6 M aqueous guanidine–HCL (0.5 μg/mL and 100 μL/well) and secondly ELISA plates were coated with 100 μL/well of the IgG fraction from an "in house" rabbit anti-human CBG, diluted in phosphate buffered saline (PBS, 5.0 μL/mL). Following coating both sets of plates were washed four times with PBS containing 0.1% Tween-20 (v/v) and "blocked" with assay buffer (PBS containing 0.1% Tween-20 (v/v) and 0.1% gelatine (w/v)), 150 μL/well for 5–10 min at 20 °C. The set of plates coated with rabbit anti-human CBG antibody were emptied by inversion and 100 μL/well of diluted pooled 3rd trimester pregnancy plasma (1:1000 in assay buffer) containing high levels of CBG (1280 nmol/L) was added to each well for 30 min at 20 °C. These plates were then washed and 50 μL/well of assay buffer was added. The RCL peptide–BTG coated and blocked plates were then emptied by inversion and 50 μL/well of assay buffer was added. Supernatants (50 μL) from the 96 well hybridoma culture plates were then added across both sets of screening plates and incubated for 60 min at 20 °C. The plates were then washed four times and sheep anti-mouse Ig-peroxidase added (100 μL/well at 1:1000 in assay buffer) for 30 min at 20 °C after which the plates were washed and substrate added. Substrate was prepared by the addition of 600 mL aqueous solution containing 8.2 g anhydrous sodium acetate and 3.6 g citric acid to 400 mL of methanol containing 270 mg of tetramethylbenzidine. Five hundred microliters of 30% H_2O_2 was finally added and the substrate stored in a dark bottle at room temperature. Colour development was terminated by the addition of 100 μL of 1.0 M HCL/well and the absorbance was read at 450 nm on a BMG Fluostar Galaxy (BMG Technologies, Germany). To avoid evaporation losses, plates were covered for all steps preceding the addition of substrate. Peptide dose–response curves and epitope mapping were also performed on RCL peptide–BTG coated plates where 50 μL of peptide in assay buffer competed with immobilised RCL peptide for antibody binding. The RCL antibodies were diluted 1:10 in assay buffer with further processing as described.

2.4. CBG ELISAs

Duplicate microtitre plates were coated using an "in house" rabbit polyclonal antibody to human CBG and processed as described previously [12]. Briefly following blocking, the plates were emptied by inversion and either 100 μL of standard or patient plasma (1:1000 dilution in assay buffer) were added for a 30 min incubation at 20 °C. The plates were washed and CBG monoclonal antibody supernatant from clone 12G2 (1:20 in assay buffer) was added to one plate and to the other supernatants from RCL hybridomas (1A9, 7D9, 8D3 or 9G12 all 1:10 in assay buffer) were added 100 μL/well for a further 30 min at 20 °C. The plates were again washed and sheep antimouse Ig-peroxidase was added for a further 30 min at 20 °C with further processing as described in the previous section. The monoclonal antibody to CBG derived from clone 12G2 does not recognise the RCL region of human CBG and hence measures total plasma CBG whereas supernatants 1A9, 7D9, 8B3 and 9G12 all recognise only intact uncleaved CBG. The CBG ELISAs performed in parallel provided measures of total and intact non-elastase cleaved CBG. The CBG ELISAs used the same standards and sample dilutions across each plate and values were interpolated on the appropriate standard curve for the antibody on that plate. Hence variation in the chromogenic response between plates was not an issue as CBG values were directly related to the standard curve on the appropriate ELISA plate.

2.5. Elastase treatment

Human leucocyte elastase was purchased from Elastin Products Co. Inc., MI, USA and reconstituted in 0.05 M Na acetate buffer, pH 5.0 containing 0.1 M NaCl and 50% glycerol and stored as a stock solution at −20 °C (19.5 units/mL) and used at final dilution of 1:100 in 0.1 M Tris–HCL buffer pH 7.5 containing 0.5 M NaCl and 0.01% NaN_3. For timed digestions of human plasma and CBG elastase, activity was terminated by the addition of a tenth volume of 10 mM PMSF in isopropanol [13].

2.6. Plasma

Intact human plasma for the elastase digestion experiments was from a local consenting individual and plasma samples for the CBG assays were from patients referred for both cortisol and CBG analyses. Purified CBG was obtained from Fitzgerald Industries, MA, USA.

2.7. Plasma free cortisol

Plasma free cortisol was measured by an established ultrafiltration/ligand binding method [1] where plasma (0.5 mL) was equilibrated with [^{3}H]-cortisol (0.1 μCi) for 30 min at 39 °C followed by centrifugation in a preconditioned ultrafiltration device (molecular weight cut-off 10,000 Da) for 10 min at 37 °C. Comparison of radioactive counts in similar volumes of ultrafiltrate and equilibrated non-centrifuged plasma provided a measure of % free cortisol which could be quantitated from total plasma cortisol [12]. Plasma free cortisol was also calculated from the levels of total plasma cortisol and CBG levels, determined by parallel ELISAs using either monoclonal antibody 12G2 (total CBG) or the RCL specific monoclonal antibody 9G12, which only recognises uncleaved (intact CBG), using the Coolens equation [14].

2.8. SDS-PAGE–Western blotting

Vertical SDS-PAGE was carried out in 10% polyacrylamide gels [15] using the Bio-Rad Mini Protean System in the presence of mercaptoethanol. Transfer to nitro cellulose was verified by the use of

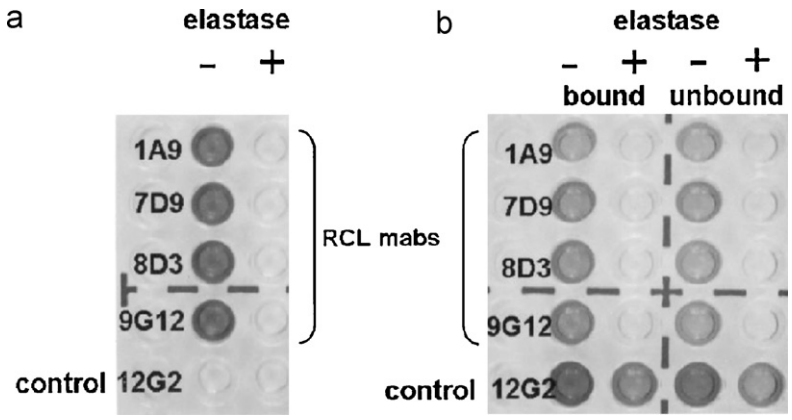

Fig. 1. Effects of human leucocyte elastase digestion on RCL peptide conjugates and on CBG. (a) Chromogenic signals from RCL antibodies (RCL mabs) and control antibody (12G2) following treatment of immobilised RCL peptide–BTG with elastase (+) or without elastase (−). (b) Chromogenic signals from RCL antibodies and control antibody (12G2) following treatment with elastase (+) and without elastase (−) of CBG bound by polyclonal CBG antibody coated wells (bound). Also shown is diluted plasma pretreated with elastase (+) or without elastase (−) prior to immobilisation by polyclonal CBG antibody coated wells (unbound).

pre-stained molecular weight markers. Following transfer nitrocellulose was blocked by incubation overnight in 5% skim milk powder and further blocked in Tris-buffered saline (TBS; 0.015 M Tris, 0.15 M NaCl, pH 7.4) containing 0.1% Tween-20 (v/v) and 1% BSA (w/v). After washing in TBS containing Tween-20 nitrocellulose was incubated with diluted hybridoma supernatant (1:10) or diluted CBG polyclonal antibody (1:500) for 60 min at 20 °C followed by either antimouse Ig-peroxidase or antirabbit Ig-peroxidase (1:5000 in TBS, containing Tween-20 and BSA). Nitrocellulose was finally washed in TBS and immunoconjugates visualised by enhanced chemiluminescence.

3. Results

Monoclonal antibody screening using the RCL peptide–BTG protocol identified 49 positive clones and of these four were positive using the immobilised CBG protocol showing these hybridoma supernatants recognised both the RCL peptide and CBG in plasma. These were all cloned twice by limiting dilution. The supernatants from these clones designated 1A9, 7D9, 8D3 and 9G12 were all isotyped as IgG2a kappa. Elastase treatment of microtitre plate wells following coating with the RCL peptide–BTG resulted in total loss of chromogenic signal (Fig. 1a) for all 4 antibodies compared to non-elastase treated wells. Similarly elastase treatment of either CBG bound to wells via immobilised CBG polyclonal antibody (bound) or diluted CBG treated with elastase prior to addition to the CBG polyclonal antibody coated plate (unbound) resulted in complete signal loss for all four RCL antibodies whereas the signal using monoclonal antibody 12G2 was only marginally affected (Fig. 1b). This slight signal loss was further investigated and found to be attributed to an elastase generated change, rather than quantitative loss, in the CBG polyclonal capture antibody. This change did not occur following PMSF inhibition of elastase activity. This is evident in Fig. 2 which shows plasma CBG levels determined using RCL monoclonal antibodies and CBG monoclonal antibody 12G2 following timed elastase digestion and termination with PMSF. Plasma levels of CBG determined using all 4 RCL monoclonal antibodies show a rapid dramatic decline compared to CBG determined with 12G2 which showed no loss even following 60 min of elastase digestion.

Fig. 3 shows a competitive ELISA with increasing doses of peptides. The immunizing RCL peptide competes with the immobilised conjugate whereas increasing doses of the synthetic peptide C-terminal to the elastase cleavage site (RCL C-terminal peptide) does not inhibit binding. Epitope mapping with a synthetic peptide library spanning the elastase cleavage site with each peptide differing incrementally by 2 amino acids is shown in Fig. 4. Wells

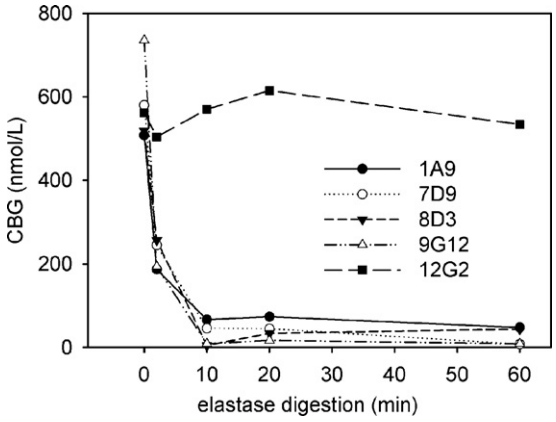

Fig. 2. Effects of human leucocyte elastase on immunoreactive human CBG. Plasma CBG levels following the timed treatment of diluted plasma with human leucocyte elastase. CBG levels were determined by the RCL antibodies (1A9, 7D9, 8D3 and 9G12) as well as with an unrelated CBG monoclonal antibody (12G2).

with TMB substrate are shown in the absence and presence of soluble peptide (100 μg/mL) where the absence of signal indicates competition for the immobilised RCL peptide–BTG conjugate by the soluble peptide for antibody binding. All four antibodies were

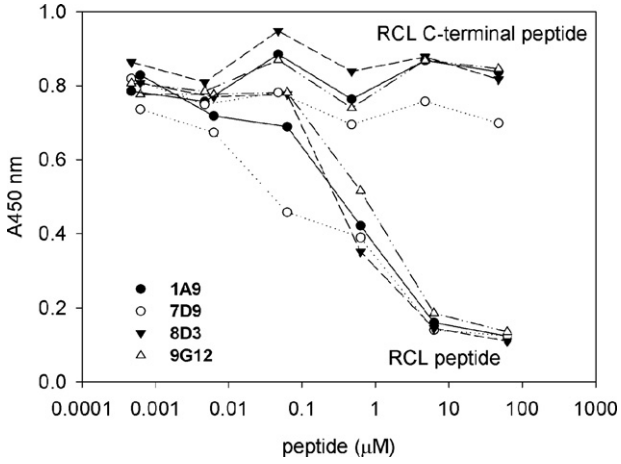

Fig. 3. Competitive effects of increasing RCL peptide levels compared to baseline on RCL peptide coated plates. Dose–response curves with increasing doses of RCL peptide and RCL C-terminal peptide on RCL peptide–BTG coated plates using RCL antibodies 1A9, 7D9, 8D3 and 9G12.

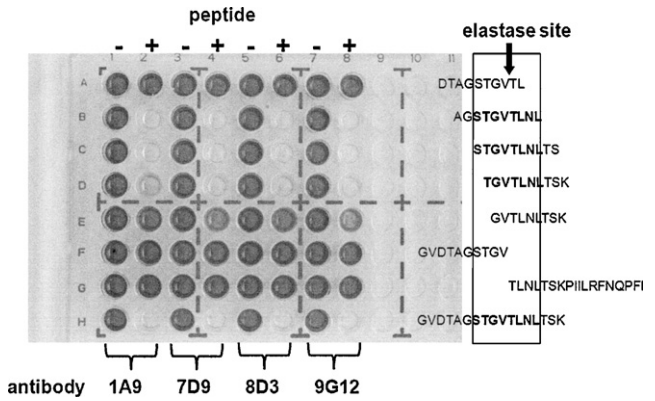

Fig. 4. Effect of various RCL peptides compared to baseline on RCL peptide coated plates. Epitope mapping using chromogenic signals from RCL antibodies 1A9, 7D9, 8D3 and 9G12 in the absence (−) or presence (+) of peptide (100 μg/mL). Peptide sequences are shown with the consensus sequence in bold within the box and the elastase cleavage site is indicated.

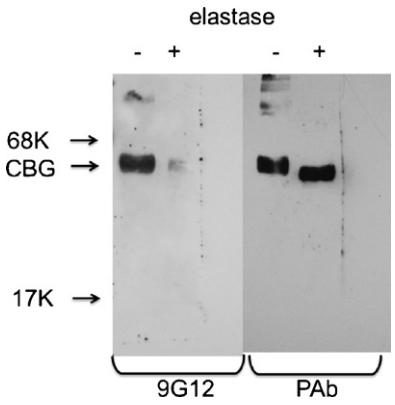

Fig. 5. Effects of human leucocyte elastase digestion on purified CBG. Western blots of purified intact (−) and elastase treated (+) CBG (0.2 μg/lane) detected with RCL antibody (9G12) or "in house" CBG rabbit polyclonal antibody (PAb). The position of molecular weight markers was indicated.

tested with identical results and the determined consensus epitope sequence which showed complete competition was STGVTLNL corresponding to Ser-341 to Leu-348 in human CBG. The peptide TGVTLNL competed less effectively for antibody binding sites.

SDS-PAGE Western blotting of elastase treated and untreated human CBG is shown in Fig. 5. Elastase treatment of CBG showed a 90% loss of intact CBG as measured by ELISA using antibody 9G12. This is consistent with the Western blot analysis where elastase treated CBG showed loss of signal compared to untreated CBG and no evidence of an altered mobility product. Conversely the use of "in house" polyclonal antibody to CBG resulted in equivalent band intensities between untreated and elastase treated CBG with the loss of the 4-kDa C-terminal fragment resulting in a higher mobility product. Attempts to carry out Western blotting using human plasma samples were confounded by the overwhelming presence of albumin and despite efforts to remove albumin Western blots remained ambiguous.

Comparison of plasma CBG levels determined by ELISA using the RCL antibody 9G12 and an unrelated monoclonal antibody 12G2 are shown in Fig. 6a with gross discordance (>20% difference) observed in 2 samples (arrowed). These 2 samples consistently showed intact CBG levels (determined by RCL antibody 9G12) less than 50% of plasma CBG levels determined by the CBG unrelated monoclonal antibody 12G2, still within or very close to the reference range (300–1300 nmol/L). These two samples had CBG levels (using 12G2) of 889 nmol/L and 1004 nmol/L and CBG levels (using RCL antibody 9G12) of 297 nmol/L and 480 nmol/L respectively (Table 1). The mean ± S.D. level of CBG in these 2 discordant samples was also determined using the other 3 RCL antibodies and was 290 ± 56 nmol/L and 460 ± 6 nmol/L respectively. We did not consider differences between total and intact CBG levels of 20% as significant as the intra- and inter-assay variation for each assay was around 10%.

Table 1 also shows the plasma levels of cortisol, calculated free cortisol levels and measured free cortisol levels of the two discordant compared to two representative concordant samples. The calculated plasma free cortisol values in the discordant samples were 2–3 times higher when CBG levels determined by the RCL antibody 9G12 were used compared to CBG levels determined using antibody 12G2. Interestingly the measured free cortisol level in the discordant samples was between the extremes, compared to concordant samples, consistent with recent crystallographic evidence that showed cleaved CBG retains lower affinity cortisol binding [9]. Plasma CBG levels of the overall sample set, determined by the RCL

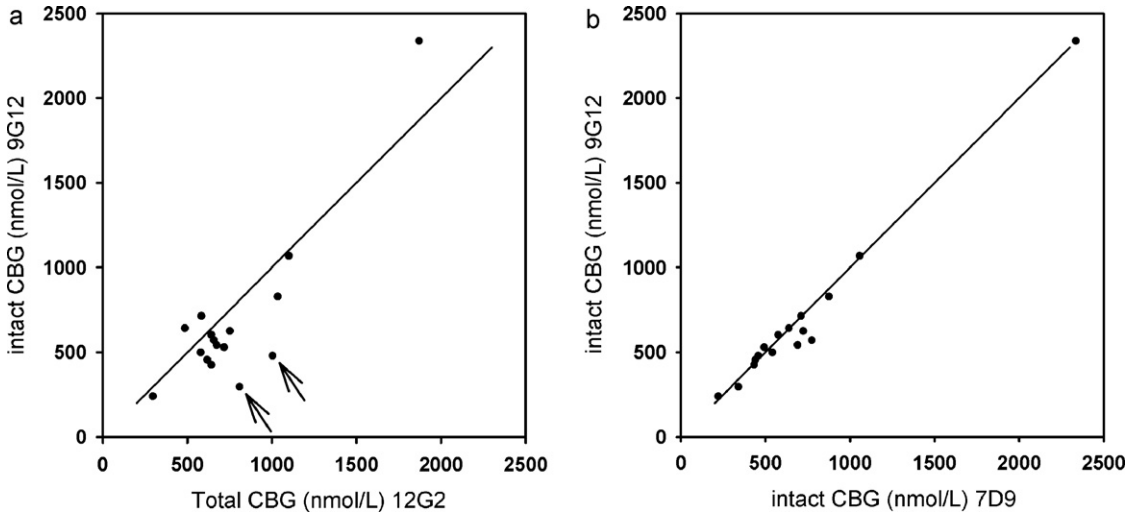

Fig. 6. Comparison of plasma CBG levels. Plasma CBG levels were determined by sandwich ELISA using RCL antibody 9G12 and CBG monoclonal antibody 12G2 on CBG polyclonal antibody coated plates. Gross discordance is shown in two samples, arrowed (a). Intact plasma CBG levels determined with two RCL antibodies (9G12 and 7D9) show concordance (b).

Table 1
Calculated levels of plasma free cortisol determined from either plasma total CBG or intact CBG and total plasma cortisol. Shown are values from two patients with discordant CBG levels and two patients with concordant CBG levels. Calculated free cortisol levels from the two samples with discordant CBG levels are shown in bold. Also shown in bold are free cortisol levels measured by ultrafiltration/ligand binding (UF/ligand binding).

	CBG total (nmol/L)	CBG intact (nmol/L)	Total cortisol (nmol/L)	Total free cortisol (nmol/L)	Intact free cortisol (nmol/L)	UF/ligand binding free cortisol (nmol/L)
Discordant sample 1	1004	480	162	**5.7**	**12.2**	**9.2**
Discordant sample 2	889	297	127	**4.9**	**14.2**	**7.6**
Concordant sample 1	1034	830	217	7.8	9.9	**10.2**
Concordant sample 2	579	500	307	24.0	28.4	**25.2**

antibodies, all show concordance and the CBG levels determined with 9G12 and 7D9 are shown (Fig. 6b).

4. Discussion

In this study it seems fairly certain that the four RCL monoclonal antibodies described are all derived from the same clone; they have the same isotype, identical behaviour following elastase treatments of the RCL peptide–thyroglobulin conjugate as well as similar peptide dose–response curves. Furthermore they show identical behaviour following elastase treatment of bound and unbound CBG and similar dose–response curves following elastase treatment of intact plasma. They also have identical epitopes which span the elastase cleavage site of the RCL domain of CBG and recognise intact CBG with recognition lost following cleavage of the exposed RCL loop.

Intact CBG has an apparent molecular weight between 52 and 58 kDa [12,16] and cleavage of the 4K peptide from CBG following elastase treatment showed an altered mobility following its release by SDS-PAGE and detection by western blotting using the CBG polyclonal antibody. This is consistent with other findings [17]. On the other hand, as expected, cleaved CBG was not recognised by the RCL monoclonal antibody as the epitope exactly spanned the elastase cleavage site. Interestingly the epitope consensus site, STGVTLNL (Ser-341 to Leu-348), also encompasses the sixth consensus carbohydrate attachment site at Asn-347, which carries complex N-glycan structures [18], and our data suggest that antibody recognition is not sterically hindered by this carbohydrate side-chain. This attachment site is also in close proximity to the elastase cleavage site (between Val-344 and Thr-345) and the rapid and complete elastase cleavage reported here also suggests adequate enzyme accessibility. This tends to counter speculation that this carbohydrate attachment site is involved in the regulation of elastase mediated steroid release from CBG [18].

The RCL antibodies reported here are likely to be useful additional tools for investigating recombinant CBG mutants with substitutions near or within the RCL loop. Elastase cleaved CBG with substitutions at the P16 residue (Glu 334 Gly or Glu 334 Ala) that show complete loss of cortisol binding [7] may not be expected to be recognised by these RCL monoclonal antibodies and other human CBG mutants with differences in ligand binding sensitivity to heat or elastase [17] are also likely candidates for the use of these novel RCL antibodies. Similarly the investigation of the temperature dependent release of cortisol from CBG could prove useful as the reactive centre loop region is temperature responsive [19].

The most significant finding implicit from this study is that both elastase cleaved and intact CBG may be present in circulation. We cannot rule out the possibility that the discordance in plasma CBG levels, in these two apparently healthy adults, was not due to a mutation resulting in partial inaccessibility to the RCL region by antibody 9G12. However we consider this possibility less likely as the only known natural CBG mutation proximal to the RCL loop, CBG Lyon Asp 367 Asn [20], is sensitive to elastase cleavage [17] implying adequate loop exposure. We were unable to test the two discordant plasma samples for elastase sensitivity as they were depleted.

The comparison and time course of plasma levels of both intact and total CBG in various disease cohorts would also be of interest as any discordance would likely have an effect on free cortisol levels in circulation and in situ glucocorticoid release. This could be clinically relevant in sepsis where overall CBG levels fall markedly with a consequent rise in free cortisol effectively enhancing activation of the HPA axis [21]. The ensuing neutrophil activation and elastase release, unless effectively inhibited, is likely to lead to CBG cleavage and, until now, the investigation of this scenario has not been possible. It is tempting to speculate that these events may reduce the levels of intact biologically active CBG with a consequent further activation of the HPA axis. CBG most likely has other functions aside from its transport role and as a determinant of free cortisol levels. Immunohistochemical methods have revealed the co-existence of CBG and oxytocin in multiple regions of the human hypothalamus leading to speculation that participates in the stress responsiveness of brain [22] and use of these antibodies could potentially provide additional evidence regarding the cellular localisation of intact natured CBG.

The current report on the generation and characterization of these novel antibodies specific for the elastase cleavage site of human CBG is topically relevant with regard to a recent review on new insights into corticosteroid-binding globulin and glucocorticoid delivery [23] and could provide an additional tool to investigate the varied mechanisms by which the body regulates glucocorticoid access to tissues.

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