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Corticosteroid-binding globulin (CBG) reactive centre loop antibodies and surface plasmon resonance interrogate the proposed heat dependent "flip-flop" mechanism of human CBG

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ABSTRACT

Corticosteroid-binding globulin (CBG) is the predominant carrier of cortisol in circulation and is a non-inhibitory member of the serpin superfamily of serine protease inhibitors. In the stressed or "S" conformation, CBG possesses an intact exposed reactive centre loop (RCL) that can be irreversibly cleaved by elastase released from activated human neutrophils whereupon it adopts a relaxed or "R" conformation. The latter conformation has decreased affinity for cortisol, allowing the release of the majority of cortisol at sites of inflammation. Recently there has been speculation that mild increments in heat such as found in pyrexia (39–40 °C) may also induce a reversible "flip-flop" of the RCL into the body of the protein structure, without cleavage, facilitating a reversible temperature-dependent release of cortisol. Here we raised a new monoclonal antibody to the RCL of human CBG and used this in concert with an existing RCL antibody and show by surface plasma resonance that, at temperatures up to 40 °C, the RCL of purified CBG and the RCL of CBG in intact plasma is accessible to these two antibodies. Together, the epitopes of these antibodies span 11 consecutive amino acids (STGVTLNLTSK) of the 18 residues of the RCL. This adequate antibody cover of the RCL sequence leads to the conclusion that the proposed temperature-dependent "flip-flop" of the RCL of CBG is doubtful.

1. Introduction

Corticosteroid-binding globulin is a monomeric circulating glycoprotein that binds up to 90% of plasma cortisol with high affinity and aids in the tissue distribution of free cortisol [1] by a variety of mechanisms. The most well-known mechanism relies on the fact that CBG is a non-inhibitory member of the serine protease inhibitor family of proteins (SERPINS) and possesses an exposed reactive centre loop (RCL) [2]. The RCL comprises 18 amino acid residues, 334–351, and spans the elastase cleavage site between Val344 and Thr345 [3]. This site can be cleaved by elastase released by activated neutrophils whereby CBG undergoes a transition from the stressed, high affinity cortisol binding state, to the relaxed state with a consequential decrease in affinity for cortisol that allows cortisol release at sites of inflammation [4]. The RCL can also be cleaved at alternative sites by chymotrypsin and the protease LasB, released by *Pseudomonas aeruginosa*, both resulting in cortisol release [5,6].

In addition to enzyme-mediated cortisol release from CBG by cleavage of the RCL it has been shown that cortisol can also be released from CBG by modest increments in temperature with a sixteen-fold decrease in cortisol affinity as the temperature increases from 35 to 42 °C [7]. This same study shows that CBG acts as a protein thermocouple that is extremely sensitive to changes in temperature releasing cortisol at temperatures typically found in the febrile state whereas cortisol release from albumin was unaffected. Both the temperature sensitivity of CBG and its affinity for cortisol have been shown to be glycosylation dependent and furthermore the difference in binding affinity for cortisol following the S to R transition is increased by glycosylation and glycosylation also reduces the cortisol association and dissociation rates [8].

This effect of temperature on alterations in cortisol binding and release from CBG has been proposed to occur via a reversible "flip-flop" of the intact reactive loop into and out of the molecule, thereby acting as a protein thermocouple [9]. We have previously raised and characterised a monoclonal antibody (9G12) which only recognises the exposed intact RCL of human CBG [10]. Here we used this antibody and raised and characterised a second RCL antibody (6B10) and used surface plasmon resonance (SPR) to

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examine whether the RCL remains exposed following increments in heat up to 40 °C.

We show by SPR that the RCL of purified CBG and the RCL of CBG in intact plasma remains accessible to both these antibodies at temperatures up to and including 40 °C. The combined epitopes of these two antibodies span 11 consecutive amino acids (STGVTLNLTSK) of the 18 residues of the RCL, including the elastase cleavage site. Given this adequate antibody cover of the RCL sequence we conclude that the proposed temperature-dependent "flip-flop" of the RCL into the protein body of CBG is questionable.

2. Material and methods

2.1. RCL monoclonal antibodies

Generation and characterisation of RCL monoclonal antibody 9G12, which spans the elastase cleavage site of human CBG, has been described [10]. In addition we raised and characterised another RCL monoclonal antibody (6B10) using a synthetic peptide to residues 333–351 of the RCL sequence, EEGVDTAGSTGVTLNLTSK-C, with a C-terminal cysteine for conjugation to BSA as the immunogen. We conjugated also this to bovine thyroglobulin for screening using the protocol described previously while for epitope mapping we used this conjugate at 10 μg/mL in 6 M aqueous guanidine HCL for coating plates and the same series of 10 mer peptides at 100 μg/mL in assay buffer [10].

2.2. CBG ELISAs

Total and intact human CBG were measured, in duplicate, by ELISA using two parallel microtitre plates coated with an "in house" rabbit polyclonal antibody to human CBG as described previously [11]. We also used a third plate also coated with "in house" rabbit polyclonal antibody to human CBG and with detection of intact CBG using the new RCL monoclonal antibody 6B10. Briefly, after "blocking" all 3 plates with assay buffer, phosphate buffered saline (PBS) containing 0.1% Tween 20 (v/v) and 0.1% gelatin (w/v), 150 μL/well, the wells were emptied by inversion and either 100 μL of CBG standard (1:1000 in assay buffer) or 100 μL of dilutions of elastase and non-elastase treated CBG (1:1000) and appropriate controls were added for 30 min incubation at 20 °C. The plates were washed and CBG monoclonal antibody supernatant from clone 12G2 (1:20 in assay buffer, 100 μL/well) was added to one plate, to measure total CBG while to the other plates, supernatants from the RCL specific CBG monoclonal antibodies 9G12 and 6B10 were added to measure intact CBG, (each 1:20 in assay buffer, 100 μL/well) for a 30 min incubation at 20 °C. The plates were again washed and either antimouse IgG1-peroxidase (1:2000 in assay buffer, 100 μL/well) was added to the plate for total CBG determination or antimouse IgG2a-peroxidase (1:1000 in assay buffer, 100 μL/well) was added to the 9G12 plate or antimouse IgM-peroxidase (1:1000 in assay buffer, 100 μL/well) was added to the 6B10 plate for intact CBG determinations. All three plates were incubated a further 15 min at 20 °C. The plates were finally washed and tetramethybenzidine (TMB) substrate was added (100 μL/well). Colour development was terminated by the addition of 1 M HCL (100 μL/well) and the absorbance was read at 450 nm. Monoclonal antibody 12G2 does not recognise the RCL of human CBG and hence can be used to measure total CBG, whereas monoclonal antibodies 9G12 and 6B10 recognise amino acid residues that span the intact elastase cleavage site of the RCL region of human CBG. Therefore, the CBG ELISAs performed in parallel provide measures of total and intact or uncleaved CBG [11].

2.3. Elastase treatment

Human leucocyte elastase was purchased from Elastin Products Co. Inc., Owensville, MO, 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 a 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 elastase digestions of

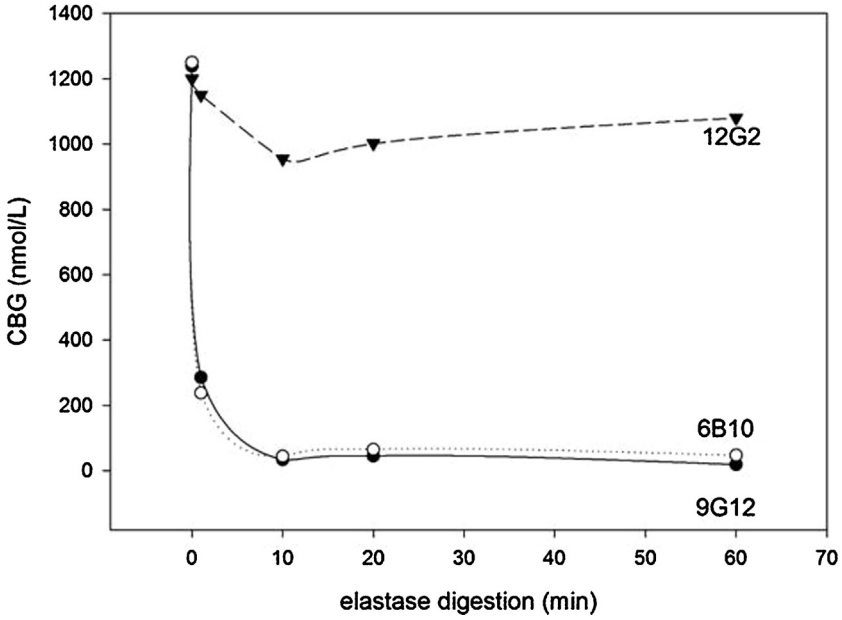

Fig. 1. Elastase affects antibody recognition of human CBG on screening peptide-thyroglobulin coated plates. Plasma CBG levels determined using RCL monoclonal antibodies 6B10 and 9G12 and the unrelated CBG monoclonal antibody 12G2 following the timed treatment of diluted plasma with human leucocyte elastase.