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MOLECULAR STUDIES OF CORTICOSTEROID BINDING GLOBULIN STRUCTURE, BIOSYNTHESIS AND FUNCTION

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Summary—Phylogenetic comparisons of the primary structure of corticosteroid binding globulin (CBG) have revealed several conserved domains that include sites for N-glycosylation and a region which probably represents a portion of the steroid binding site. The major site of CBG biosynthesis in adults is clearly the liver, and the human CBG gene promoter contains sequence elements that interact with liver-specific transcription factors. Low levels of CBG gene expression have been detected in other tissues, and these may be important for fetal development during late gestation when hepatic CBG mRNA levels are low. Studies of the ontogeny of CBG biosynthesis in the rat have also indicated that plasma CBG levels may be influenced by a more rapid clearance of the protein during pubertal development. Analyses of the structural organization and chromosomal location of the human CBG gene have further confirmed its close relationship with the serine proteinase inhibitors, and suggests that CBG, α_1-proteinase inhibitor and α_1-antichymotrypsin evolved relatively recently by gene duplication. The functional significance of this relationship has been examined and our studies suggest that a specific interaction between CBG and elastase on the surface of neutrophils may represent a physiologically important event that promotes the delivery of glucocorticoids to these cells at sites of inflammation.

INTRODUCTION

A high-affinity transport protein for glucocorticoids has been identified in all vertebrate blood samples studied [1, 2], and is generally referred to as corticosteroid binding globulin (CBG).† The biochemistry and physiology of the protein have been examined in great detail, and this information has been summarized in a comprehensive monograph on steroid–protein interactions by Westphal [2]. In essence, CBG is usually characterized as an ~50–60 kDa monomeric glycoprotein, with a single steroid binding site that interacts preferentially with biologically active glucocorticoids, but it may also have a relatively high affinity for progesterone in some species [2]. Apart from a few notable exceptions [2], the CBG binding site in most mammals is almost entirely occupied by glucocorticoids, and it binds as much as 90% of these steroids in the blood circulation [3].

In general, studies of the production of CBG under different physiological conditions, or after hormone treatment, have been restricted to measurements of its levels in blood [2]. Although these studies have provided valuable information, the changes observed may simply be attributed to an alteration in clearance rather than the biosynthesis of the protein. The major site of CBG synthesis has always been assumed to be the liver [4], but the protein has also been detected in a variety of other tissues using both biochemical and immunocytochemical approaches [5–8]. It is not known whether this is due to local synthesis or accumulation from the blood, and has prompted speculation about the physiological significance of CBG in glucocorticoid target tissues [3]. Indeed, the consensus has always been that CBG functions exclusively as a plasma transport protein for glucocorticoids, but improvements in the purification of CBG [9] have allowed more detailed studies of its functional properties, and these have been reviewed recently [10].

In 1987, the isolation of a cDNA for human CBG revealed the primary structure and

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†CBG is also referred to as transcortin.

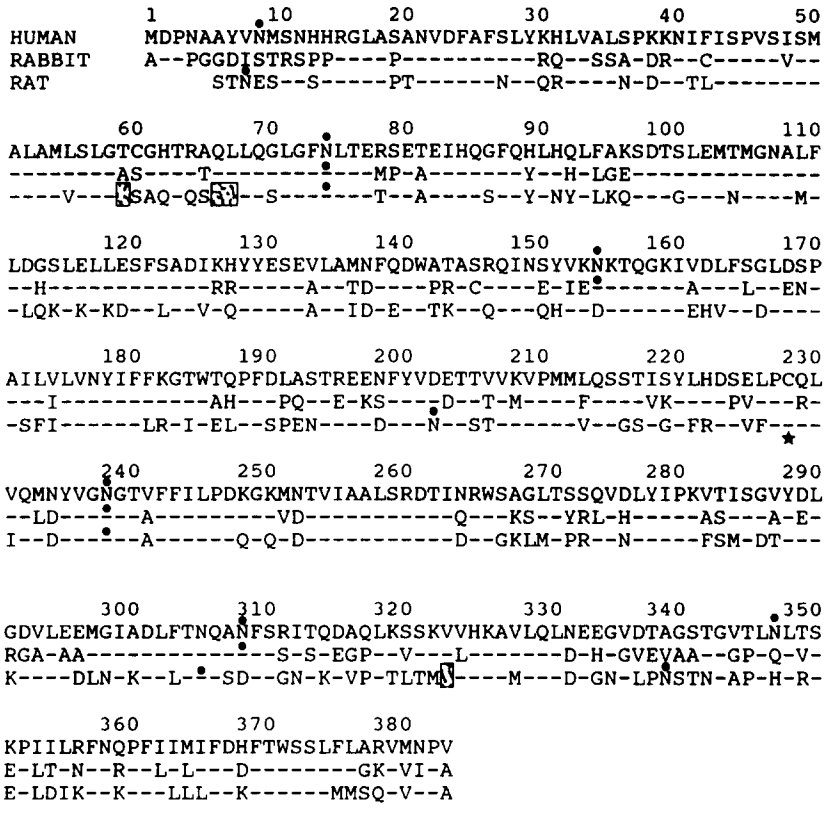

Fig. 1. Phylogenetic comparison of CBG primary structure. Amino acid (single letter code) sequences are aligned with respect to the human CBG sequence, and identical amino acids in other sequences are indicated by a dash. Deletions in the rat sequence are represented by boxes. Consensus sites for N-glycosylation are shown (●) as is the conserved cysteine residue (★).

molecular composition of the protein [11], and was followed by the isolation and characterization of the human CBG gene [12], which has recently been assigned to chromosome 14 [13]. Collectively these studies have provided new insight into the structure and origins of CBG and its gene. But perhaps the most remarkable discovery has been the identification of a structural relationship between CBG and several members of the serine proteinase (serpin) superfamily; the most notable examples being α_1-proteinase inhibitor (A1-PI), α_1-antichymotrypsin (ACT) and thyroxin binding globulin [11]. The availability of a human CBG cDNA has also allowed the cloning of CBG cDNAs from several other species [14, 15], and phylogenetic comparisons of CBG primary structure are beginning to provide additional information about structurally and functionally important domains. Much of this information has been reviewed recently [16], and this overview will concentrate on some of the more important data that have emerged from our recent studies of the structure, biosynthesis and function of CBG in various species.

PHYLOGENETIC COMPARISONS OF THE MOLECULAR PROPERTIES OF CBG

The cDNA-deduced primary structures of human, rat* and rabbit CBG are presented in Fig. 1. Human and rabbit CBG both contain 383 amino acids and their calculated polypeptide molecular weights are 42,646 and 42,326, respectively. By contrast, rat CBG contains only 374 amino acids and has a polypeptide molecular weight of 42,229. The degree of sequence similarity between CBGs from different species (60–70%) is relatively poor when compared to many other proteins. In this regard, it should also be noted that the rat CBG sequence is not only shorter at its amino-terminus, but there are also three places where deletions of one or more amino acids occur, when compared to the human and rabbit proteins. These deletions are located in regions that are not well conserved, and are therefore probably not biologically very important. Although it is not immediately apparent how or why these deletions have

*An error in the published cDNA sequence of rat CBG [14] has been detected which converts residues 141 and 142 in the rat sequence from *Thr Arg* to *Asn Gln*.

occurred, one of them is adjacent to an intron/exon junction in the human gene [12].

Small differences in polypeptide composition do not entirely account for the relatively large species differences in the apparent molecular size (M_r) of CBG, when examined by SDS–PAGE [17], and certainly do not explain the size heterogeneity that has also been observed within individual blood samples [18]. These differences are probably due to variations in carbohydrate composition, and knowledge of the primary structure of CBG in different species has enabled us to identify the sites at which N-linked carbohydrates may be attached. Detailed carbohydrate analyses of human CBG have indicated that the molecule comprises an average of five N-linked oligosaccharide chains [19], and the cDNA-deduced human CBG sequence contains six consensus sites for N-glycosylation [11]. It is therefore important to note that amino-terminal sequence analysis of human CBG has demonstrated that a carbohydrate chain is attached to the Asn at position 9 in the mature polypeptide sequence [20]. Like human CBG, the rat protein also has a consensus site for N-glycosylation close to its amino-terminus, but this does not appear to be utilized [17]. Furthermore, when considered together with the obvious lack of conservation in amino-terminal sequences between species, and the absence of a glycosylation site in the amino-terminal region of rabbit CBG (Fig. 1), it is likely that this carbohydrate chain in the human sequence is of limited importance.

Although there is little additional information about other sites of glycosylation, it is interesting that all four consensus N-glycosylation sites in the rabbit CBG sequence are located in exactly the same positions in the human sequence, and it is therefore likely that these sites are all utilized. However, it is possible that some sites are only partially utilized, and we have recently obtained evidence that the site located within the carboxy-terminal region of the molecule is not always glycosylated, and may also have different carbohydrate structures attached to it [21].

When the human, rat and rabbit sequences are compared, it is also evident that only two of the N-glycosylation sites have been retained throughout evolution. Furthermore, these consensus sites are located in highly conserved regions of the protein, and may therefore be functionally very important. Although the significance of the carbohydrate structures that decorate CBG is not understood, they may certainly influence the biological half-life of the protein, and play an important role in any interaction between CBG and possible receptors on the plasma membranes of different tissues [22, 23].

Phylogenetic comparisons of the steroid binding activity of CBG have indicated that the affinity and specificity of the steroid binding site vary between species [2], but it invariably displays a preference for the biologically most important glucocorticoid in a given species. For example, human CBG has a higher affinity for cortisol than corticosterone while rat CBG has a higher binding affinity for corticosterone. The relative affinities of CBG for glucocorticoids in different species also vary by at least an order of magnitude; an extreme example being the difference between the steroid binding affinity of CBG for cortisol in the Old World primates when compared to New World monkeys [24]. We therefore anticipate that comparisons of the primary structure of CBG between species may help identify regions that constitute the steroid binding domain.

Previous physicochemical analyses of the binding site have indicated that the steroid is located approximately 25 Å from the surface of human CBG, with the D-ring and the C21 side chain of the steroid molecule being directed towards the surface of the protein [25], and affinity-labelling with 6-bromo-progesterone has located a cysteine in the steroid binding site [26, 27]. It is therefore important to note that human CBG contains only two cysteine residues, which are not linked in the native molecule [27], while the rat sequence only contains one located in an identical position (residue 228) in both the human and rabbit sequences (Fig. 1). Furthermore, this cysteine resides within an extended sequence of approximately 40 residues which are highly conserved between all three species. This region is poorly conserved when compared to other closely-related serpins, e.g. Al-PI [11], and this may be significant because it is known that several highly-conserved domains within the serpin superfamily are essential for maintaining their characteristic tertiary structure [28]. It would therefore appear that this region represents a functionally important domain in CBG that is not shared by other serpins, such as a steroid binding site.

When the primary structures of human, rat and rabbit CBG are compared, there is a very

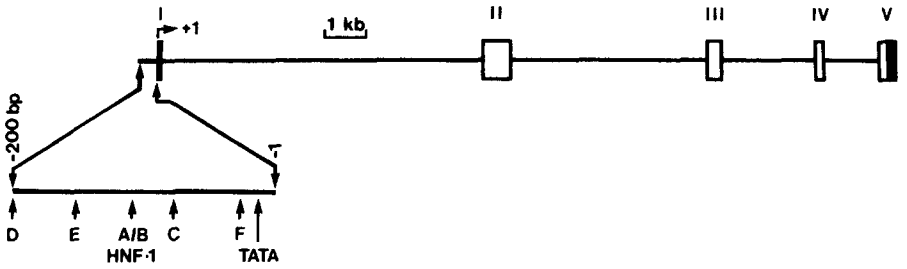

Fig. 2. Promoter and transcription unit for hepatic CBG mRNA in humans [12]. The human CBG gene is comprised of five exons (boxes), numbered I–V distributed over a 19 kb region of genomic DNA. Both 5′ and 3′ untranslated regions are shown (filled boxes). A 200 base pair region 5′ of the transcription start site (+1) has been enlarged to indicate the location of possible transcription factor binding sites (A–F) in the CBG gene promoter, including HNF-1, and were identified by comparison to the mouse albumin gene promoter [36].

poorly conserved region between amino acids 330 and 360 with respect to the human sequence. Interspecies variability in this region has also been noted with other serpins [29], and may be significant because this region of the serpin superfamily is known to interact with target serine proteinases [30]. The reason for this is unknown, but it has been attributed to evolutionary pressures that have forced the serpins to adapt to serine proteinases produced by species-specific, infectious organisms [29]. An alternative explanation is that the serine proteinases themselves have undergone rapid evolutionary change in different environments, and that the serpins have co-evolved to maintain their functional role. This region of the molecule is also relatively poorly conserved between closely-related serpins, and it is known that very minor variations in the composition of this region can radically alter the affinity and specificity of this site for serine proteinases [31]. It is therefore remarkable that CBG and Al-PI are both excellent substrates for neutrophil elastase [21, 32].

BIOSYNTHESIS OF CBG

The liver has been identified as the major site of CBG biosynthesis in several mammalian species [11, 14, 15] and the protein is produced and secreted by hepatocytes in culture [33–35]. The transcription unit responsible for CBG mRNA in the human liver has been characterized (Fig. 2), and has revealed the presence of a promoter region that contains several highly conserved DNA sequence elements that are necessary for efficient liver-specific expression of the mouse albumin gene [36]. Interestingly, both CBG and albumin gene promoters contain a consensus binding sequence for the hepatocyte-specific transcription factor, HNF-1 [37]. This factor is the major determinant of the expression of a number of other genes in the liver [38], in addition to the albumin [39] and A1-PI [40] genes, and is probably important in the development of the liver phenotype [37]. It is therefore likely that HNF-1 is an essential part of the transcriptional apparatus responsible for the expression of the CBG gene in the liver.

The availability of species-specific cDNAs for CBG has enabled us to detect CBG mRNA by Northern blot analyses of spleen and ovary RNA from adult rabbits [15] and kidney and testis RNA from a rhesus monkey [11] and a CBG cDNA has been cloned from a human lung library [11]. Although the levels of CBG mRNA in these tissues are relatively very low when compared to adult liver samples, it should be noted that similar amounts of CBG mRNA are present in the kidney and liver of fetal rabbits during late gestation [15]. In contrast, a comprehensive Northern blot of rat tissues revealed CBG mRNA only in the liver [14] but *in situ* hybridization of rat lung has indicated that these transcripts are present in discrete cell populations surrounding bronchiolar epithelial cells [16 41]. It therefore appears that very low levels of CBG mRNA can be detected in extrahepatic tissues of adult animals, but only a small proportion of cells within a tissue may express the CBG gene. By analogy, although the human A1-PI gene is expressed predominantly in the

Table 1. Serum CBG cortisol binding capacities of various species

Species	pmol cortisol bound/ml serum
Bovine	386
Guinea-pig	353
Human	606
Pig	88
Rabbit	706
Rat	1103
Sheep	243

From Ref. [1].

liver, A1-PI mRNA has also been detected in macrophages [42]. In addition, expression of a human A1-PI transgene has also been detected in liver, kidney, macrophages, and other cell types, at levels which parallel the expression of the endogenous mouse A1-PI gene [43]. These studies have indicated that transcription of the human A1-PI transgene in the liver and kidney involves the same initiation site, while the expression of this gene in other cell types utilizes a macrophage-specific promoter [43]. Therefore the production of CBG mRNA in extra-hepatic tissues may not necessarily involve a single cell type.

Serum concentrations of CBG vary between species (Table 1), and there are remarkable species-differences with respect to diurnal variation [44, 45]; response to hormonal treatment [46 47]; and the sex of the animal [4, 45, 48]. During pregnancy, maternal serum CBG concentrations increase during late gestation in humans [4, 48, 49] and other mammals [2], and have been shown to decline just prior to term in several animal models [50–52]. Although the increments observed vary considerably between species [2], they generally reflect changes in hepatic CBG mRNA levels [14, 15] and are therefore probably due to alterations in CBG biosynthesis rather than clearance [15, 53].

Hepatic CBG mRNA levels in fetal rats and rabbits are much higher than in their corresponding mothers early in the last third of gestation, and this is remarkable because the liver is largely composed of erythropoietic cells at this stage of development [54]. After this point in time, the biosynthesis of CBG in the fetal rat and rabbit declines, even though maternal serum levels are still increasing [15, 55], and this supports the concept that CBG production is regulated independently in fetal and maternal compartments [35, 53]. Although exogenous glucocorticoids reduce serum CBG levels in several species [46, 56], the levels of both total and free glucocorticoids are very low in both rat [57] and rabbit [52] fetuses at about 70% of gestational age, and are therefore unlikely to initiate a decrease in fetal CBG biosynthesis observed during late gestation in these species. However, this decline in fetal circulating CBG levels ultimately increases serum levels of free glucocorticoids and may contribute to developmental events, such as lung maturation [58]. It should also be noted that this developmental profile of fetal CBG production is not observed in all species, and the sheep is a notable example in which fetal CBG biosynthesis appears to increase markedly just prior to term [59], and this is thought to contribute to the onset of parturition [60].

At term [2], or shortly thereafter [59], serum CBG concentrations are remarkably low in all species, and hepatic CBG mRNA levels are barely detectable in neonatal rats and rabbits [14, 15]. Recently, we have studied the ontogeny of CBG biosynthesis in the rat, and have observed that hepatic CBG mRNA levels increase by at least 5-fold between 1 and 2 weeks of age, with males livers containing approximately one-third less CBG mRNA than the livers of age-matched females [55]. This sexual dimorphism in CBG gene expression has been noted previously [14], and has been attributed to androgen imprinting of pulsatile growth hormone secretion patterns [61, 62]. By three weeks of age, adult CBG mRNA values are attained, while serum CBG concentrations do not reach adult values until three weeks later, and we have shown that this may be due to a difference in the clearance rate of CBG in infant rats when compared to adult animals [55].

FUNCTIONAL RELATIONSHIP BETWEEN CBG AND THE SERPINS

It is becoming increasingly obvious that the relationship between CBG and other members of the serpin superfamily is not simply confined to similarities at the primary structural level. For instance, we have recently demonstrated that the structural organization of the human CBG, A1-PI and ACT genes is very similar [12], and that the CBG gene is actually more closely related to the A1-PI gene than any other serpin gene identified so far. In addition, *in situ* hybridization studies of human metaphase chromosome spreads with a human CBG cDNA probe have localized the human CBG gene to a region within chromosome 14 (q31–32.1) that also contains the human A1-PI and ACT genes [13]. Collectively these data indicate that these three genes have evolved relatively recently by a process of gene duplication, and that this may have occurred to accommodate refinements of a physiologically important function in the vertebrate species. It is therefore perhaps not merely coincidental that A1-PI and ACT play important roles in controlling the activities of serine proteinases during inflammation [30], and that CBG represents the major transport

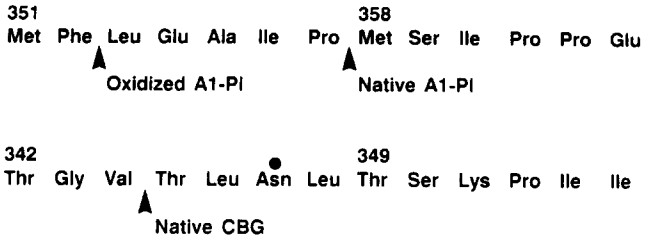

Fig. 3. Elastase-cleavage sites in oxidized [69] and native [31] A1-PI and native CBG [32]. A consensus site for N-glycosylation is present in the CBG sequence (●) and does not influence elastase cleavage when utilized [21].

protein for one of natures most important anti-inflammatory agents; namely the glucocorticoid hormones. These observations further support the concept that an interaction between CBG and a serine proteinase at sites of inflammation may result in the delivery of glucocorticoids to cells involved in the inflammatory process [11].

It has been known for many years that CBG responds as an acute phase negative protein during inflammation [63], and that serum CBG levels drop rapidly during either artificially-induced inflammation [63] or the onset of infectious diseases [64, 65]. Although hepatic biosynthesis of CBG appears to be reduced considerably during acute inflammation [64, 66], degradation of CBG at sites of inflammation, and clearance of cleaved CBG, may also account for the rapid decline in serum CBG levels that occurs relatively early in the inflammatory process [64, 65]. More recently it has been demonstrated that CBG is specifically cleaved by neutrophil elastase [21], and that this promotes a conformational change in the protein [32] which disrupts the steroid binding site, and releases steroid hormone [21, 32]. Furthermore, it should be noted that the initial cleavage of CBG by elastase occurs in virtually the same position as the site at which elastase cleaves oxidized A1-PI (Fig. 3). Although the kinetics of this interaction between CBG and elastase have not yet been examined in detail, it may well be characterized by a K_d of $\sim 10^{14}$M if the reaction resembles that which occurs between A1-PI and elastase [31].

Although the physiological implications of these observations have not been clearly defined, we have recently demonstrated that CBG interacts specifically with elastase on the surface of neutrophils taken from patients with acute inflammation [67], and this process does not appear to involve the internalization of CBG [67]. Furthermore, it is clear that this interaction effectively reduces the steroid binding activity of CBG, and presumably results in the release of glucocorticoids directly to the activated neutrophils. It is likely that this process occurs mainly at sites of inflammation, where large amounts of glucocorticoids are required to control the destructive potential of activated neutrophils. Moreover, it also probably occurs as one component of a very tightly regulated sequence of events, and the following scenario may be envisaged.

During inflammation, recruitment and activation of neutrophils results in the production of large amounts of superoxide radicals and serine proteinases, and these are both essential for the killing and degradation of infectious material. The production of superoxide radicals is also known to destroy the ability of A1-PI to inhibit elastase, and thereby allows this serine proteinase to function more efficiently in a local context. On the other hand, CBG is not influenced by the presence of superoxide radicals (unpublished data) and retains its ability to interact with neutrophil elastase, which appears to associate with the surface of these cells once they have been activated [67]. The CBG is then rapidly cleaved by elastase and provides a mechanism for the delivery of relatively large amounts of glucocorticoids to the activated neutrophils. At this stage in the inflammatory reaction, the glucocorticoids down-regulate neutrophil activity by decreasing the production of chemotactic factors, and other products that are involved in the inflammatory process, such as prostaglandins [68].

It is also possible that this type of mechanism is not strictly limited to inflammatory diseases, and may also be relevant during normal physiological events such as growth and development. In this context, we have recently observed that the serum clearance of CBG during postnatal development in the rat occurs much more rapidly than in adult animals [55]. Since it has been suggested that serine proteinases play an important role in the normal process of

tissue remodelling and development [30], it is possible that CBG may promote the delivery of glucocorticoids to sites where rapid growth and tissue remodelling are occurring as a result of normal development. Thus providing a mechanism for the natural suppression of local inflammatory reactions that may occur during this process.

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