J. steroid Biochem. Vol. 35, No. 1, pp. 61–65, 1990
Printed in Great Britain.

0022-4731/90 $3.00 + 0.00

CHARACTERIZATION OF THE MURINE CORTICOSTEROID BINDING GLOBULIN: VARIATIONS BETWEEN MAMMALIAN FORMS

LENA NYBERG,[3]* LYUBEN N. MAREKOV,[1]† ILONA JONES,[3] GUNILLA LUNDQUIST[1] and HANS JÖRNVALL[1,2]

[1]Department of Chemistry I, Karolinska Institutet, S-104 01 Stockholm, [2]Center for Biotechnology, Huddinge Hospital, S-141 86 Huddinge and [3]Department of Clinical Chemistry, College of Veterinary Medicine, S-750 07 Uppsala, Sweden

(*Received* 26 *August* 1989; *received for publication* 22 *August* 1989)

Summary—Corticosteroid binding globulin (CBG) from term-pregnant mouse serum was isolated and characterized by peptide analysis after treatment with CNBr and Lys-specific protease, respectively. Amino acid sequence analysis of six segments, covering 189 of 383 positions in different regions of the protein, showed unexpectedly low overall homology (60%) to the indirectly deduced human amino acid sequence previously reported. However, some segments displayed a greater resemblance to their human counterparts. Differences were observed in at least two of six potential glycosylation sites. The nature of electrophoretic CBG variants and their immunological properties are described.

INTRODUCTION

Corticosteroid binding globulin (CBG) is a serum protein specifically binding glucocorticoids. It has been suggested that CBG influences the ratio of free to bound hormone and transports corticosteroids to target tissues [1, 2]. The protein has been described from virtually every vertebrate species studied [3]. It is structurally related to serine protease inhibitors of the serpin type [4], such as thyroxine-binding globulin [5]. Molecular weights of CBG generally range between 50,000–60,000 in most species; the proteins are glycosylated (roughly 20% carbohydrate) and have similar amino acid compositions [6].

Several authors have reported the *N*-terminal amino acid sequence of human CBG [5, 6], and the whole human CBG structure has been deduced from the corresponding cDNA [4]. Extensive differences have been noted between the *N*-terminal segments of rat and human CBG [9], and immunological results have revealed a low degree of cross-reactivity [9]. The limited amount of information available at present cannot be readily interpreted and little is known about the degree of relationship between CBGs of different species.

The previously published purification procedure [10] has now been improved, and a preparation suitable for structural analysis has been obtained. Selected peptide analysis of two different digests revealed structures of different regions of the mouse CBG molecule. The analysis allowed the internal parts of the mouse and human CBGs to be compared.

*Author to whom correspondence should be addressed.
†Present address: Institute of Molecular Biology, 1113 Sofia, Bulgaria.

EXPERIMENTAL

CBG purification

CBG was isolated from term-pregnant mouse serum according to a protocol utilizing exclusion chromatography on Sephacryl-S-200 and fast protein anion exchange chromatography [10]. To separate electrophoretic variants or unrelated proteins, an additional fast protein ion exchange chromatography step was introduced. Thus, to the CBG fraction obtained at pH 5.0 [10] an equal volume of 20 mM Tris–HCl, pH 8.8, was added, and the mixture was immediately loaded again on Mono Q HR 5/5. The column was washed with 10 mM Tris–HCl, pH 8.5, and developed at 1 ml/min with a 20 min gradient of 0.6 M NaCl 0–100% in the same buffer. Purity was checked by SDS/polyacrylamide gel electrophoresis (PhastSystem, Pharmacia). Corticosteroid binding capacity was assayed with [^{3}H]corticosterone (105 Ci/mmol, New England Nuclear) as described previously [10].

Antiserum

Polyclonal antibodies to murine CBG were raised in rabbits. CBG was prepared as described [10], and 0.7 mg was emulsified in 1 ml of Freund's complete adjuvant and injected i.d. Two booster injections of 0.3 mg immunogen in Freund's incomplete adjuvant, were given at three weeks interval. Animals were bled and sera collected 12 days after the last booster. Antisera were utilized for crossed immunoelectrophoresis [11].

Structural analysis

Purified CBG (10 nmol) was reduced with diethiothreitol (500 nmol) in 6 M guanidine–HCl, 0.4 M

Tris–HCl, 2 mM EDTA, pH 8.15 (2 h, 37°C) and carboxymethylated with ^{14}C-labelled, neutralized iodoacetic acid (1500 nmol; 1.5 h, room temperature; 2,400 cpm/nmol). Reagents were removed by extensive dialysis against distilled water, and separate samples were used for determination of the total composition, direct *N*-terminal sequence analysis, and peptide analysis of internal segments. Peptides were obtained by treatment of the preparation with CNBr (0.2 g/ml in 70% formic acid at room temperature for 24 h), or with Lysobacter lysine-specific protease (Boehringer Mannheim; ratio protease/protein of 1:40, in 0.1 M ammonium bicarbonate for 4 h at 37°C). The proteolytic digests were purified by reversed phase high-performance liquid chromatography on an LKB Ultropac C_{18} column, with a gradient of acetonitrile in 0.1% trifluoroacetic acid [12]. Amino acid compositions were determined (Beckman 121 M amino acid analyzer) after hydrolysis for 24 h at 110°C with 6 M HCl/0.5% phenol in evacuated tubes. Sequence degradations were carried out in an Applied Biosystems 790A gas phase sequencer, with reverse phase HPLC (Hewlett–Packard 1090 instrument) for phenylthiohydantoin identification [13].

RESULTS

Electrophoretic variants of CBG and immunological properties

The CBG preparation obtained by fast protein liquid chromatography at pH 5.0 [10] revealed two bands when subjected to SDS gel electrophoresis (data not shown). An additional separation step was therefore introduced (see Experimental), which resolved the material into two separate fractions (Fig. 1).

Both fractions exhibited corticosteroid binding capacity, although to different extent (Fig. 1) and had largely similar total compositions, which also correspond to those of other CBG preparations (Table 1). Because of its higher binding capacity, peak one was chosen for further analysis. This fraction yielded a single band on SDS gel electrophoresis and direct *N*-terminal sequence analysis indicated that no contaminating structures were present.

Serum from pregnant mice produced a single immunoprecipitin line against rabbit antimouse CBG (Fig. 2) in a crossed immunoelectrophoresis analysis (see Experimental).

Structural analysis

Attempts to obtain an *N*-terminal amino acid sequence by direct sequencer degradation gave no result. This suggests that the protein is blocked, most probably by *N*-terminal acetylation, which is a common protein modification [14].

Separate batches of the carboxymethylated CBG were cleaved by treatment with CNBr and Lys-specific protease, respectively. Peptides obtained were purified by reverse phase HPLC. In total, 15 peptides were subjected to sequencer degradations, which together defined 189 residues from different regions of the protein. The two sets of peptides gave several consecutive or overlapping fragments; thus we were able to determine the sequence for positions 109–182 and 274–314. The amino acid sequences determined were long enough to allow alignment with the indirectly deduced human CBG (Fig. 3).

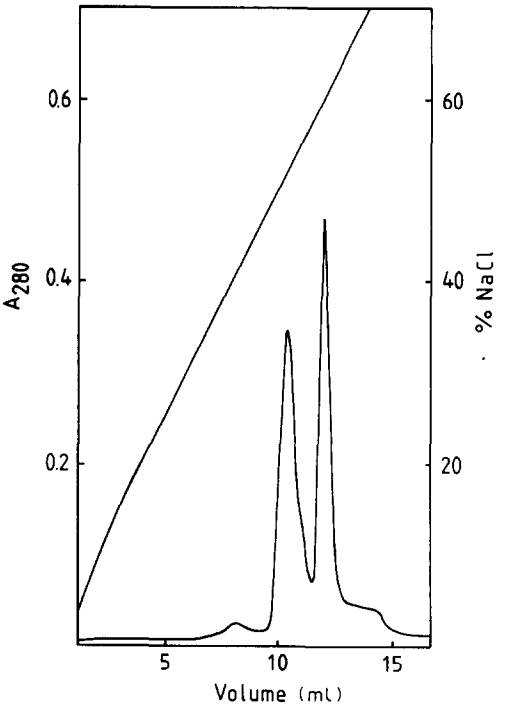

Fig. 1. Fast protein liquid chromatography of purified CBG at pH 8.5. The preparation obtained at pH 5.0 was reapplied on a Mono Q column and eluted with 10 mM Tris–HCl, pH 8.5 with a 0.6 M NaCl 0–100% gradient for 20 min (1 ml/min). Fractions were tested for corticosteroid-binding capacity (see Experimental): peak 1 = 19.7 nmol/l, peak 2 = 8.4 nmol/l.

Table 1. Amino acid composition of CBG from different species

	Mouse	Rat	Human
Cys	0.5	0.7	0.5
Asx	12.6	11 8	11.0
Thr	7.0	7.0	6.8
Ser	9.1	8.2	9.4
Glx	10.0	12.7	8.9
Pro	4.8	5.1	3.1
Gly	5.6	6.4	5.5
Ala	5.9	7.0	6.8
Val	5.4	6.1	7.3
Met	3.2	2.4	3.7
Ile	5.4	3.9	5.7
Leu	11.3	10.3	11.5
Tyr	3.0	3.0	2.9
Phe	4.6	4.8	6.0
Trp	ND	—	1.0
Lys	6.7	6.1	4.4
His	2.1	2.1	2.9
Arg	3.0	3.0	2.6
Sum	100.5	100.9	100.6

Left column shows values (mol%) now determined. Compositions of rat [9] and human [4] CBG are given for comparison.

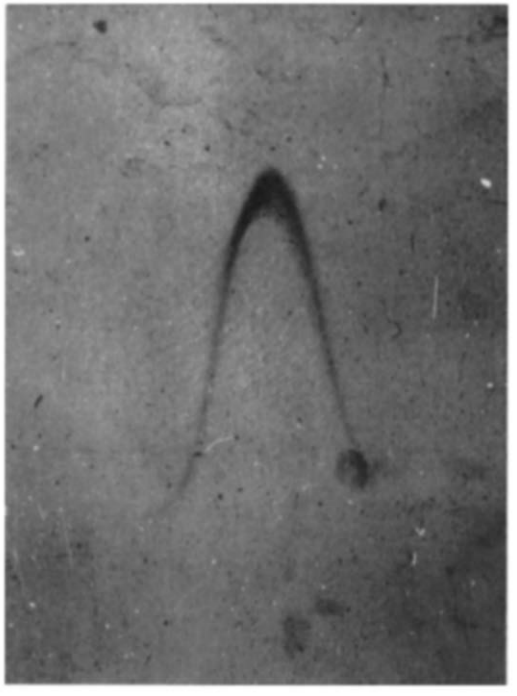

Fig. 2. Crossed immunoelectrophoresis of pregnant mouse serum in agarose gel containing 1% antiserum against mouse CBG. Serum was electrophoresed 15 V/cm for 50 min in the first dimension and 2 V/cm for 15 h in the second dimension.

```
Hu- CBG    MDPNAAYVNMSNHHRGLASANVDFAFSLYKHLVALSPKKNIFISPVSISM
mouse
rat              STNES---S----PT------N--EC----NCD--TL-

51
ALAMLSLGTCGHTRAQLLQGLGFNLTERSETEIHQGFQHLHQLFAKSDTSLEMTMGNALF
                                  M.-S--R----A-                M-

111
LDGSLELLESFSADIKHYYESEVLAMNFQDWATASRQINSYVKNKTQGKIVDLFSGLDSP
-LQN-K-KD--L--T-------A-TIPSK--TK-GE---NH--A------EHVV-L---G

171
AILVLVNYIFFKGTWTQPFDLASTREENFYVDETTVVKVPMMLQSSTISYLHDSELPCQL
QT-I-P----L-                                        -V--G----FR--AI---

231
VQMNYVGNGTVFFILPDKGKMNTVIAALSRDTINRWSAGLTSSQVDLYIPKVTISGVYDL
                        --------.-----          M---V  -FSM-DT---

291
GDVLEEMGIADLFTNQANFSRITQDAQLKSSKVVHKAVLQLNEEGVNTAGSTGVTLNLTS
Q---ADV--K----A-SD-ADT-K                --M---T-GN-LP-N-N-PPV  -P

351
KPIILRFNQPFIIMIFDHFTWSSLFLARVMNPV
     KY-E----LA-
```

Fig. 3. Summary of the amino acid sequence analysis of mouse CBG. (Standard-one-letter abbreviations). The structures determined (middle line) are shown below the indirectly deduced human structure [4] (top line). Also included (bottom line) is the *N*-terminal structure of rat CBG [9] now aligned with the human CBG. Numbers shown refer to human CBG starting at Met-1. Dashes in middle and bottom lines indicate proven residue identities with the top line. Dots at positions 79 and 259 of the middle line refer to residues *not unambiguously defined but likely to represent* Ser as shown. Bars indicate peptides analysed and show the region investigated. The preceeding amino acid was deduced from specific cleavage as: the M for CNBr fragments and K for peptides obtained by cleavage with Lys-specific protease.

The two proteins were found to be homologous: For segment 109–182, 42 of 74 residues were identical between mouse and human CBG. An entirely conserved segment was found around position 250 (Fig. 3); however, most regions had several substitutions. Combined, the mouse CBG peptides investigated had residues identical with the human CBG at only 111 of 189 positions (60%) studied. This proportion is low compared with many other proteins from these two species.

The segments analysed were also compared to protein sequences in the NBRF-PIR Protein Sequence Database (release 16.0) [15]. Regions 109–182, 212–229 and 251–263 were apparently related to the α_1-antichymotrypsin (45, 58 and 42%, respectively) and to α_1-antitrypsin to an extent of 47 and 38% (segment 109–182 and 281–315, respectively). Similarities between segments 79–90 and 109–182 and the thyroxine-binding globulin were found (75 and 35%, respectively). The region 281–315 showed also a clear homology with thyroxine-binding globulin (44%). The same segment was further found to have a structure like a fragment of the mouse protease inhibitor contrapsin (43%), as expected from the serpin relationships.

DISCUSSION

In this study, we conducted the first protein analysis of internal peptide segments of the murine CBG. This allows a comparison with the indirectly deduced amino acid sequence of the human CBG [4], and a judgement of the partial sequence previously reported for the rat counterpart [9]. We have also presented information about the apparent molecular heterogeneity upon SDS–PAGE analysis.

Two electrophoretic forms of murine CBG were separated. The material in peak 1 apparently represents pure CBG. The material in peak 2, may be a variant. The two forms exhibit corticosteroid-binding capacity (although to different extents) and have similar total amino acid compositions. Further, a single precipitin line is obtained on crossed immunoelectrophoresis. Hence, we find it likely that the two peaks represent isoforms of CBG. Human CBG is also composed of two corticosteroid-binding forms eluted separately on Mono Q [16], and electrophoretic variants of CBG have earlier been described [9, 17, 18]. However, these electrophoretic forms of mouse CBG were not detected in a previous study [10]. At the present stage, we believe that the increased resolution now is explained by the use of better electrophoretic techniques. However, both fractions have to be further investigated in order to establish the relationship.

Alignment with the predicted amino acid sequence of human CBG revealed clear resemblance for all seven segments analysed and their corresponding human counterparts. However, local regions of strict sequence correlation are interrupted by considerably less conserved stretches. For example, peptides at positions 109–119, 133–146 and 334–346 show a pronounced divergence, whereas those at positions 120–132, 147–160 and 251–263 display prominent similarity. Presumably, the three closely related regions represent a segment(s) in the CBG molecule of comparatively high functional importance. The complete conservation of the amino acid residues in the hydrophobic region position 260, may indicate the presence of an internal segment possibly involved in steroid-binding which would appear compatible with a well-preserved substrate specificity. Alternatively, the occurence of both constant and variable peptides may indicate the presence of two somewhat different but homologous protein chains corresponding to the two fractions detected.

The degree of overall residue identity between the mouse and human CBG molecules is 60%. This value is lower than the degree of similarity observed between the amino acid sequences of several other proteins for these species [19]. Furthermore, no serologic cross-reactivity has been reported [20, 21], indicating that conservation is low in CBG. Apparently, large segments of the CBG molecule have been subjected to a fairly extensive evolutionary divergence and presumably comprise regions of modest functional importance.

The comparisons further show specific differences in the *N*-terminal segments. Native mouse CBG is apparently blocked, probably by *N*-terminal acetylation, while rat and human CBG appear to have free α-amino groups [4–6, 9]. However, it may be noted that the rat and human CBGs previously reported to be different [9] are indeed also homologous, as now shown in (Fig. 3), but starting at non-equivalent positions. Presumably, the mouse CBG has yet another *N*-terminal structure, susceptible to α-aminoacethylation [22].

No significant homology was found between the mouse CBG and other steroid-binding globulins in the NBRF protein database. However, the mouse CBG showed clear homology to thyroxine-binding globulin, α_1-antichymotrypsin and α_1-antitrypsin. This is in accordance with earlier reports on human CBG and reflects the serpin family assignment [4]. The CBG also revealed a similarity to mouse contrapsin. Thus, mouse CBG is clearly a member of the same family.

Finally it should be noted, that on the basis of the structures determined, the results suggest differences in sites for *N*-linked glycosylations, i.e. two out of six potential Asn-Xxx-Thr/Ser sites in mouse CBG differ from those in human CBG. Asn-308 is replaced by Asp, and the sequence Asn-Leu-Thr starting at position 347 of the human protein is replaced by Asn-Leu-Pro in the mouse protein (Fig. 3).

In conclusion, our results show that the CBG protein structure is variable and that clear relationships exist between the mammalian CBGs characterized, including those with previously concluded

dissimilar *N*-terminal segments. Interestingly, a high degree of variability, also in important segments, has been noticed for another anti-protease structure, that of ovalbumin [23]. The high degree of sequence variability within CBG and the differences in potential glycosylation sites suggest comparatively low restrictions on the protein, and explain the immunological lack of cross-reactivity between different CBGs.

Acknowledgements—We are grateful to Anne Peters for skillful assistance. This work was supported by grants from the Swedish Council for Forestry and Agricultural Research, the Swedish Cancer Society (project 1806) and Karolinska Institutet (fellowship to LNM).

REFERENCES

1. Siiteri P. K., Murai J. T., Hammond G. L., Nisker J. A., Raymoure W. J. and Kuhn R. E.: The serum transport of steroid hormones. *Rec. Prog. Horm. Res.* **38** (1982) 457–510.
2. Westphal U.: *Monographs on Endocrinology. Steroid-Protein Interactions II.* Springer-Verlag, Berlin, Heidelberg (1986).
3. Seal U. S. and Doe R. P.: Corticosteroid binding globulin. Isolated from plasma of diethylstilbestrol treated men. *J. Biol. Chem.* **237** (1962) 3136–3140.
4. Hammond G. L., Smith C. L., Goping I. S., Underhill D. A., Harley M. J., Reventos J., Musto N. A., Gunsalus G. L. and Bardin C. W.: Primary structure of human corticosteroid binding globulin, deduced from hepatic and pulmonary cDNAs, exhibits homology with serine protease inhibitors. *Proc. Natn. Acad. Sci. U.S.A.* **84** (1987) 5153–5157.
5. Le Gaillard F., Han K. K. and Dautrevaux M.: Caractérisation et propriétés physico-chimiques de la transcortine humaine. *Biochemie* **57** (1975) 559–568.
6. Fernlund P. and Laurell C.-B.: A simple two-step procedure for the simultaneous isolation of corticosteroid binding globulin and sex hormone binding globulin from human serum by chromatography on cortisol-sepharose and phenyl-sepharose. *J. Steroid Biochem.* **14** (1981) 545–552.
7. Flink I. L., Bailey T. J., Gustafson T. A., Markham B. E. and Morkin E.: Complete amino acid sequence of human thyroxine-binding globulin deduced from cloned DNA: close homology to serine antiproteases. *Proc. Natn. Acad. Sci. U.S.A.* **83** (1986) 7708–7712.
8. Rosner W.: Recent studies on the binding of cortisol in serum. *J. Steroid Biochem.* **3** (1972) 531–542.
9. Kato E. A., Hsu B. R-S. and Kuhn R. W.: Comparative structural analyses of corticosteroid binding globulin. *J. Steroid Biochem.* **29** (1988) 213–220.
10. Nyberg L. and Jones I.: A simple method for the purification of murine corticosteroid binding globulin: characterization of a monomer. *J. Steroid Biochem.* **29** (1988) 341–346.
11. Clarke H. G. and Freeman T.: Quantitative immunoelectrophoresis of human serum proteins. *Clin. Sci.* **35** (1968) 403–413.
12. Jeffery C., Cederlund E. and Jörnvall H.: Sorbitol dehydrogenase. The primary structure of the sheep-liver enzyme. *Eur. J. Biochem.* **140** (1984) 7–16.
13. Kaiser R., Holmquist B., Hempel J., Vallee B. L. and Jörnvall H.: Class III human liver alcohol dehydrogenase of: a novel structural type equidistantly related to the class I and class II enzymes. *Biochemistry* **27** (1988) 1132–1140.
14. Persson B., Flinta C., von Heijne G. and Jörnvall H.: Structures of N-terminally acetylated proteins. *Eur. J. Biochem.* **152** (1985) 523–527.
15. Sidman K. E., George D. G., Barker W. C. and Hunt L. T.: The protein identification resource (PIR). *Nucl. Acids Res.* **16** (1988) 1869–1871.
16. Heubner A., Belovsky O., Müller W., Grill H. J., Manz B., Juchem M. and Pollow K.: Application of liquid-liquid partition chromatography in the simultaneous purification of sex-hormone-binding globulin and corticosteroid-binding globulin. *J. Chromat.* **397** (1987) 419–434.
17. Favre G., Le Gaillard F., Mattret-Tunion M. H., Dumur V. and Dautrevaux M.: Physico-chemical properties and evidence for electrophoretic variants of rat transcortin. *Biochimie* **66** (1984) 361–369.
18. Mickelson K. E., Harding G. B., Forsthoefel M. and Westphal U.: Steroid-protein interactions. Human corticosteroid-binding globulin: characterization of dimer and electrophoretic variants. *Biochemistry* **21** (1982) 654–660.
19. Jörnvall H., Persson B. and Jeffrey J.: Characteristics of alcohol/polyol dehydrogenases. The zinc-containing long-chain alcohol dehydrogenases. *Eur. J. Biochem.* **167** (1987) 195–201.
20. Hsu B. R., Kato E. A., Raymoure W. J. and Kuhn R. W.: A homologous radioimmunoassay for guinea pig corticosterone-binding globulin. *Proc. Soc. Exp. Biol. Med.* **185** (1987) 243–249.
21. Sueda K., Seo H. and Matsui N.: Comparative study of primates' transcortin: immunoreactivity and steroid-binding activity. *Endocr. Jap.* **33** (1986) 143–150.
22. Flinta C., Persson B., Jörnvall H. and Heijne G.: Sequence determinants of cytosolic N-terminal protein processing. *Eur. J. Biochem.* **154** (1986) 193–196.
23. Laskowski Jr. M., Kato I., Ardelt W., Cook J., Denton A., Empie M. W., Kohr W. J., Park S. J., Parks K., Schatzley B. L., Schoenberger O. L., Tashiro M., Vichot G., Whatley H. E., Wieczorek A. and Wieczorek M.: Ovomucoid third domains from 100 avian species: isolation, sequences, and hypervariability of enzyme–inhibitor contact residues. *Biochemistry* **26** (1987) 202–221.