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THE GLUCOCORTICOID RECEPTOR IN RAT LIVER

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Glucocorticosteroids, in similarity to all other active steroid hormones, exert their biological effect *via* a soluble receptor protein in the target cell. Twenty years ago Jensen and Jacobson first demonstrated the specific binding of [^{3}H]-estradiol by rat uterus, a target organ for estrogens [1]. Since then, a great deal of information has been obtained with regard to the mechanism of action of steroid hormones. A model for the mechanism of action of steroid hormones, in particular glucocorticosteroids is shown in Fig. 1.

There are separate proteins for androgens, estrogens, glucocorticosteroids, mineralocorticosteroids, gestagens and vitamin D, respectively. The steroid, in this case a glucocorticosteroid, passes through the cell membrane of the target cell, presumably by passive diffusion, into the cytoplasm. Here the steroid binds to specific receptor proteins and the complex is then translocated into the nucleus where it interacts with DNA. Before the nuclear translocation or interaction with DNA can take place *in vitro*, the GR* must be "activated". "Activation" can be achieved by incubating the complex at 20° or by incubation at 0° in the presence of 0.12 M NaCl or KCl. Activation probably incurs a conformational change of the steroid receptor complex and presumably occurs immediately after the binding of steroid to the receptor *in vivo*. Activation of the complex greatly stabilizes the interaction of the steroid with the receptor protein.

After the translocation of the complex into the nucleus, it binds to the genome, presumably binding to the DNA of the specific genes regulated by the hormone. This interaction results in the specific transcription of these genes with a resulting synthesis of the specific enzymes induced. For instance, the addition of glucocorticosteroids to liver cells results in the induction of tyrosine amino-transferase and tryptophan oxygenase activities.

*Abbreviations: GR, glucocorticoid receptor complex; MMTV, mouse mammary tumour virus; SDS, sodium dodecyl sulphate.

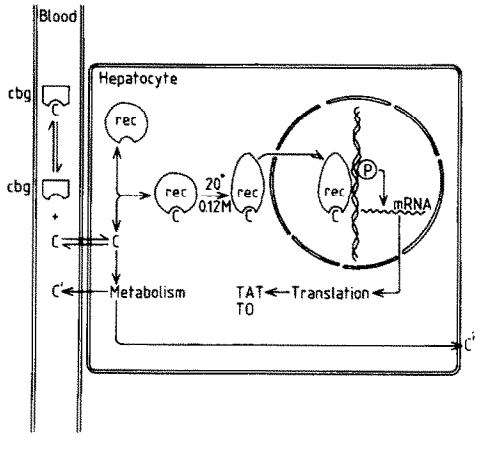

Fig. 1. Model for the mechanism of action of glucocorticosteroids. Abbreviations: C, corticosteroid; cbg, corticosteroid-binding globulin; rec, glucocorticosteroid receptor protein; P, DNA-dependent RNA-polymerase; TO, tryptophan oxygenase; TAT, tyrosine aminotransferase; C', corticosteroid metabolites (inactive).

PURIFICATION OF RAT LIVER GR

Recently, several groups have been successful in purifying the glucocorticoid receptor using a variety of different techniques. We have purified the rat liver GR by sequential chromatography on calf thymus DNA-cellulose with an intermediate step involving heat-activation [2]. A flow diagram for the principle of the purification is shown in Fig. 2. After preparation of the rat liver cytosol and incubation with [^{3}H]-triamcinolone acetonide, the cytosol is passed through a phosphocellulose and a calf thymus DNA-cellulose column in order to remove all DNA-binding

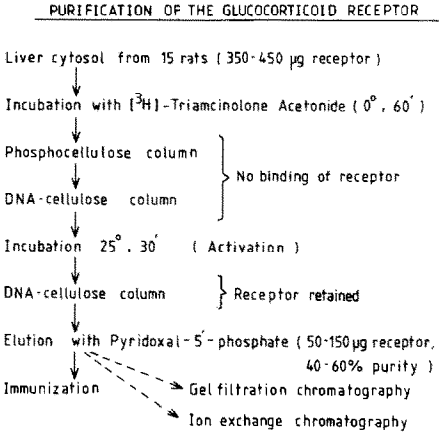

Fig. 2. Flow diagram for the purification of the rat liver glucocorticoid receptor by sequential chromatography on calf thymus DNA-cellulose with intervening activation. The GR can be eluted from the second DNA-cellulose column by either 10 mM pyridoxal 5'-phosphate or 25 mM $MgCl_2$. The GR can be further purified by ion exchange chromatography on DEAE-Sepharose, gel filtration on Sephadex G-200 or by SDS-gel electrophoresis. For further details see Refs. [2, 10, 14].

proteins. During these steps it is important to work as fast as possible and to keep the receptor preparation well cooled in order to minimize proteolysis and activation of the GR. After passage through the first DNA-cellulose column, the receptor preparation is heat-activated and re-chromatographed on DNA-cellulose to which it will now bind. After thorough washing of the column, the GR can be eluted from the column using 10 mM pyridoxal 5'-phosphate, 0.18 M NaCl or 25 mM $MgCl_2$. Elution by 0.18 M NaCl results in an unstable GR with rapid dissociation of the steroid from the receptor. The purity of the receptor preparation after elution with pyridoxal 5'-phosphate is about 50% based on the radioactivity eluted and the protein quantitation. However, pyridoxal 5'-phosphate binds competitively to the DNA-binding site of GR, although it can be displaced by incubation with 100 mM dithiothreitol [3].

Milgrom *et al.* [4] have previously shown that magnesium ions inhibit the binding of GR to DNA and Stevens *et al.* [5] used magnesium ion for elution of GR from cell nuclei. We found 25 mM $MgCl_2$ to be optimal for elution of GR from the second DNA-cellulose column when considering both purity (63 ± 19%; N = 6; mean ± S.D.) and recovery (34 ± 6%; N = 7). Furthermore, the 25 mM $MgCl_2$-eluted GR could be applied on a DEAE-Sepharose column and be rapidly eluted with a linear salt gradient resulting in a considerable purification. The recovery in the DEAE-Sepharose step was 66 ± 7% (N = 5). The purity of DEAE-Sepharose purified GR was about 80% according to densitometry scanning of SDS-electrophoresis gels.

Physico-chemical analysis of the purified GR resulted in the following data. The molecular weight according to SDS-gel electrophoresis was 94,000 daltons. The Stokes radius as determined by gel filtration on Sephadex G-200 was 6.0 nm. The sedimentation rate was 3.4 S in 0.15 M KCl and 3.6 S in the absence of KCl. The isoelectric point was 5.8. According to the radioactivity measurements, the protein quantitation and the molecular weight, there was one steroid-binding site per receptor molecule.

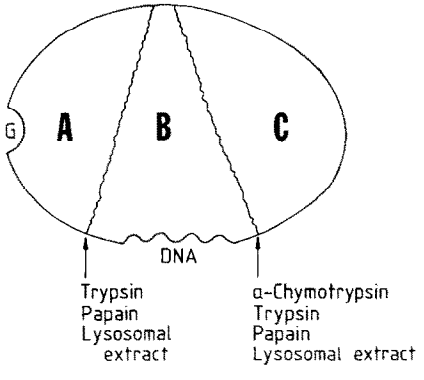

Fig. 3. Model for the functional domains of the GR. Domain A contains the steroid binding site, domain B contains the calf thymus DNA binding site, and domain C contains the immunological determinant(s). For further details see Refs. [9, 10].

FUNCTIONAL DOMAINS OF THE GLUCOCORTICOID RECEPTOR

Limited proteolysis of rat liver GR, both in crude cytosol [6–10] and purified GR [11], has been shown to give rise to two specific fragments of GR. These fragments have been analysed with regard to several biological functions common to steroid receptors namely steroid- and DNA-binding and also immunoactivity. Using limited proteolysis of GR with trypsin or α-chymotrypsin it was possible to define three separate domains containing the three above described functions (Fig. 3). Similar findings have been described for the androgen receptor [12], as well as for other steroid receptors [13].

Limited proteolysis of GR in crude cytosol preparations (Stokes radius 6.1 nm) by trypsin or α-chymotrypsin results in a fragment of GR with Stokes radius 3.3–3.7 nm (depending on which matrix gel filtration is performed). Further proteolysis with trypsin, but not α-chymotrypsin, results in a fragment of GR with Stokes radius 1.9 nm. Extracts from purified liver lysosomes have the same effect as trypsin [14].

Antibodies against the purified rat liver GR have been raised in seven different rabbits [15, 16] as well as monoclonal antibodies [17]. All seven polyclonal antibodies, as well as the monoclonal antibodies, have been found to interact with the same part of GR, namely domain C (Fig. 3).

A summary of the data from the limited proteolysis of GR in crude cytosol as well as purified GR [6–11] is as follows:

The 6.1 nm complex (the native GR) has a molecular weight of 94,000 daltons. It contains the steroid-binding site (domain A), the calf thymus DNA-binding site (domain B) and interacts with the antibodies raised against purified GR (domain C; immunoactivity).

The 3.3–3.6 nm fragment binds both steroid (domain A) and calf thymus DNA (domain B). In fact it has higher affinity for calf thymus DNA than the 6.1 nm complex. It does not interact with the antibodies raised against the purified GR. The molecular weight according to SDS-gel electrophoresis is 39,000 daltons.

The 1.9 nm fragment binds only steroid (domain A) and not calf thymus DNA. It has no immunoactivity. The molecular weight is 27,000 daltons.

Initial cleavage of the receptor results in two major immunoactive fragments, as determined by SDS-gel electrophoresis, with molecular weights of 49,000 and 45,000 daltons respectively (domain C). The Stokes radius is 2.6 nm. These two fragments can be further degraded into a smaller fragment with molecular weight 27,000 daltons and Stokes radius 1.4 nm.

SPECIFIC INTERACTION OF GR WITH DNA

In collaboration with Yamamoto's group, especially Payvar and Firestone, the specific interaction of the purified GR with the mouse mammary tumour virus (MMTV) gene has been studied [3, 18, 19].

Specific interaction of the purified GR with the MMTV gene has been studied using two methods.

Fig. 4. MMTV receptor binding domain. The diagram shows an intact MMTV element oriented in the normal configuration of the integrated provirus [24]; restriction sites for PstI (P), SacI (S) and EcoRI (R) are noted. The wavy line shows the sites of initiation and polyadenylation of the primary transcript; boxes defined by unbroken lines depict restriction fragments containing high affinity binding sites for purified glucocorticoid receptors; box defined by broken line contains a lower affinity binding site as measured in the nitrocellulose filter assay; the heavy dashed line marks a PstI:EcoRI fragment known to contain at least one high affinity receptor binding domain (this region has not yet been investigated in detail); filled circles depict receptor binding sites mapped by electron microscopy; open circles in the right hand long terminal repeat sequence (LTR) are sites inferred from the left hand LTR but have not been confirmed directly. Figure from Ref. [19].

The first method is a nitrocellulose filter assay. The cloned DNA fragments are end-labelled with ^{32}P, incubated with purified GR and then passed through a nitrocellulose filter. The protein-bound DNA fragments are retained on the filter and can be eluted by treatment with SDS. The DNA fragments retained are then analyzed by agarose-gel electrophoresis and autoradiography. By using a variety of restriction enzymes, one can map regions of the gene that interact with the purified GR. Competition experiments wth unlabelled DNA fragments, both from the MMTV gene and other sources such as bacteriophage T4 or salmon, demonstrate that the interaction of the purified GR with MMTV DNA is specific.

Interaction of the hormone receptor complex with MMTV DNA has also been investigated by electron microscopy [18, 19]. This method provides an independent assessment of specific binding by the receptor, allowing a provisional estimate of the number and position of receptor binding sites within a given region. The results are fully consistent with those from the filter binding assay.

Figure 4 shows our current view, showing that the receptor selectively associates with at least five widely separated regions of the MMTV DNA element; electron microscopic analyses of two of the domains reveal that each harbours multiple closely-spaced binding sites.

Cloned fragments of MMTV DNA that contain binding domains but lack the MMTV promoter were introduced into cultured cells by cotransfection with cloned HSV thymidine kinase (tk) DNA [18, 19]. Phenotypic tk^+ transformants were obtained in each of the three cases studied, using three different regions of MMTV DNA, each containing receptor binding sites. In all three cases the tk^+ transformants displayed dexamethasone inducible expression. This suggests that either the internal fragments contain hormone-responsive promotors that are cryptic in the intact provirus, or that they somehow enhance transcription from nearby cellular or vector promoters.

Thus, one can demonstrate specific interaction of rat liver GR with the MMTV gene, a gene that is induced by glucocorticosteroids in a receptor dependent manner [20, 21]. The regions of DNA containing the specific sites of interaction for DNA can be fused with other sequences and in this manner confer regulation of this sequence by glucocorticosteroids [18, 19, 22, 23].

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REFERENCES

1. E. V. Jensen and H. I. Jacobson, *Rec. Prog. Hormone Res.* **18**, 387 (1962).
2. Ö. Wrange, J. Carlstedt-Duke and J.-Å. Gustafsson, *J. biol. Chem.* **254**, 9284 (1979).
3. F. Payvar, Ö. Wrange, J. Carlstedt-Duke, S. Okret, J.-Å. Gustafsson and K. R. Yamamoto, *Proc. natn. Acad. Sci. U.S.A.* **78**, 6628 (1981).
4. E. Milgrom, M. Atger and A. Bailly, *Eur. J. Biochem.* **70**, 1 (1976).
5. J. Stevens, Y.-W. Stevens, J. Rhodes and G. Steiner, *J. Natl. Cancer Inst.* **61**, 1477 (1978).
6. J. Carlstedt-Duke, J.-Å. Gustafsson and Ö. Wrange, *Biochim. biophys. Acta* **497**, 507 (1977).
7. Ö. Wrange and J.-Å. Gustafsson, *J. biol. Chem.* **253**, 856 (1978).
8. Ö. Wrange, J. Carlstedt-Duke and J.-Å. Gustafsson, in *Proteases and Hormones* (Ed. M. K. Agarwal), pp. 141–157. Elsevier/North-Holland Biomedical Press, Amsterdam (1979).
9. J. Carlstedt-Duke, S. Okret, Ö. Wrange and J.-Å. Gustafsson, *Proc. natn. Acad. Sci. U.S.A.* **79**, 4260 (1982).
10. J. Carlstedt-Duke, Ö. Wrange, S. Okret, J. Stevens, Y.-W. Stevens and J.-Å. Gustafsson, in *Gene Regulation by Steroid Hormones* (Eds. A. K. Roy and J. H. Clark), Vol. 2, pp. 151–180. Springer, New York (1983)
11. Ö. Wrange, S. Okret, M. Radojcic, J. Carlstedt-Duke and J.-Å- Gustafsson, *J. biol. Chem.* 259, in press (1984).
12. E. M. Wilson and F. S. French, *J. biol. Chem.* **254**, 6310 (1979).
13. M. R. Sherman, L. A. Pickering, F. M. Rollwagen and L. K. Miller, *Fedn. Proc.* **37**, 167 (1978).
14. J. Carlstedt-Duke, Ö. Wrange, E. Dahlberg, J.-Å. Gustafsson and B. Högberg, *J. biol. Chem.* **254**, 1537 (1979).
15. S. Okret, J. Carlstedt-Duke, Ö. Wrange, K. Carlström and J.-Å. Gustafsson, *Biochim. biophys. Acta* **677**, 205 (1981).
16. S. Okret, *J. Steroid Biochem.* **19**, 1241 (1983).
17. S. Okret, A.-C. Wikström, Ö. Wrange, B. Andersson and J.-Å. Gustafsson, *Proc. natn. Acad. Sci. U.S.A.* **81**, in press (1984).
18. F. Payvar, G. L. Firestone, S. R. Ross, V. L. Chandler, Ö. Wrange, J. Carlstedt-Duke, J.-Å. Gustafsson and K. R. Yamamoto, *J. Cell. Biochem.* **19**, 241 (1982).
19. K. R. Yamamoto, F. Payvar, G. L. Firestone, B. A. Maler, Ö. Wrange, J. Carlstedt-Duke, J.-Å. Gustafs-

son and V. L. Chandler, *Cold Spring Harbor Symp. Quant. Biol.* **47**, 977 (1983).
20. G. M. Ringold, K. R. Yamamoto, J. M. Bischop and H. E. Varmus, *Proc. natn. Acad. Sci. U.S.A.* **74**, 2879 (1977).
21. J. R. Grove, B. S. Dieckmann, T. A. Schroer and G. M. Ringold, *Cell* **21**, 47 (1980).
22. F. Lee, R. Mulligan, P. Berg and G. Ringold, *Nature* **294**, 228 (1981).
23. A. L. Huang, M. C. Ostrowski, D. Beraud and G. L. Hager, *Cell* **27**, 245 (1981).
24. G. M. Ringold, P. R. Shank, H. E. Varmus, J. Ring and K. R. Yamamoto, *Proc. natn. Acad. Sci. U.S.A.* **76**, 665 (1979).