Review Letter

PHYSICAL CHARACTERISATION OF CYTOPLASMIC GLUCO- AND MINERALO-STEROID RECEPTORS

M. K. AGARWAL+

INSERM U-36, 17 Rue du Fer-à-Moulin, Paris 75005, France

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1. Introduction

Delineation of corticosteroid hormone action is not only one of the few means available to understand the organisation and expression of the complex mammalian genome but it also provides important cues for current therapy in a wide variety of clinical disorders. The accepted sequence of events is based around the concept where the steroid ligand bound to its cytoplasmic carrier moves into the nucleus and associates itself with the acceptor sites in the target. The present paper is limited to a review of current literature on physical characterisation of the cytosol vector specific for the gluco- and the mineralocorticoids. This excludes detailed consideration of other steroid binders (viz., albumin, ligandin, transcortin) as well as of receptors for other classes of hormones (estradiol, progesterone, testeosterone) although they have been taken into consideration when relevant. Since a current research on Medlar revealed more than 300 papers during the three preceding years, reference is most often made to a number of existing reviews [1–3] and books [4,5], where possible for both gluco- [1,4,5] and mineralo- [2–5] groups of hormones. Literature citation is not meant to establish priorities but to lead the reader to an appropriate departing point for further thought.

2. Technical considerations

A currently accepted sequence of events in the mechanism of corticoid hormone action consists of the entry of the lipophilic steroid molecule into the cell cytoplasm with subsequent binding to specific high affinity (K_d 10^{-10} or close to it), low capacity (therefore saturable) receptors. This complex is thereafter 'activated' by a number of factors (temperature, ionic strength), presumably in the cytoplasm. The activated complex is finally said to penetrate the nuclear membrane and to interact with the acceptor sites in the chromatin and/or DNA as a prerequisite for modulation of the organ specific response. The role of high capacity (thus nonsaturable), low affinity (K_d approx. 10^{-9}) serum binders (transcortin, albumin) is not clear in this sequence of events (discussed later). Thus, a receptor is the immediate requirement for tissue response whereas a binder is an agent capable of transporting the ligand but not necessarily of eliciting the desired reaction.

Since the concept of a receptor, by definition, presupposes the presence of a macromolecule within any given target capable of responding to a specific external stimulus by timely, sequential and orderly alteration in a group of processes, a speculative presumption explained corticoid response as a 'one hit' type of mechanism where possibly only one single site may be involved for all grades of agonist and antagonist activity, depending upon the affinity of the ligand for its vector. The ease and rapidity of Scatchard type of analysis further channelled all experimental effort towards the elucidation of a receptor a priori considered homogeneous and unitary by the very nature of definition. More recent comparative work with various techniques based on saturation and competition has revealed discrepancies in the interpretation of results obtained with equilibrium binding, especially since the latter must be disturbed by the addition of

+Maître de Recherches au CNRS

charcoal prior to estimation of saturation characterstics. Physical separation and purification, thus, were clearly needed to establish the nature of the receptor and it is to this aspect the sections below are addressed.

3. Glucocorticoid receptor

Density gradient centrifugation has been one of the earliest and the most time honoured tools available to the molecular biologist. Application of this method to receptor separation was beset with two major difficulties. First, lengthy centrifugation periods resulted in dissociation of the bound ligand although synthetic steroid (triamcinolone, dexamethasone) receptor complexes [8] could withstand dissociation more readily than natural steroid–receptor complexes [9–11]. Second, this procedure is based upon separation by sedimentation coefficients of the macromolecule varying in S values between 3 S for the thymocyte receptor [8] to 5.5 S in the fibroblast [12] and does not permit resolution of activated versus inactive forms of the receptor. Association into larger 7–8 S forms in presence of low salt, and their dissociation into smaller 4 S units with high salt buffers could however be achieved in the lung with density gradient centrifugation [13]. Fractions of smaller molecular weight, Stoke's radius and sedimentation coefficients were observed under conditions that favoured proteolytic cleavage in rat liver cytosol [14].

Among numerous gels, Sephadex beads of graded sizes were the first available to the biochemist. With this technique of molecular weight filtration, a monomer approx. mol. wt 67 000 was estimated for rat liver cytosol binder [15–17] which could polymerise into complexes of higher molecular weight reaching as high as 200 000 for rat liver [9–11] and 600 000 for mouse fibroblasts [12]. Upto five different components could be observed in adult chicken liver cytosol [18] although only one was present in 16 day old chick embryo cytosol [19]. Only one of these five entities appeared to exhibit receptor activity as judged by binding to nuclei and the contribution of others was not clear nor was it assessed whether polymerization could explain this multiplicity since rechromatography of individual peaks was not attempted [18].

From the foregoing, techniques used for receptor separation based mostly on component molecular weights led inadvertently to the concept of a homogeneous, unitary monomer capable of polymerisation into larger, dissociable units. With advance in technology of charge dependent fractionation, receptor polymorphism in vitro could be made evident in presence of different steroids, ions and resins and is summarised below; selected properties have been condensed in table 1.

The most detailed analysis with DEAE-cellulose-52 resins was conducted by the author beginning as early as 1970 with rat liver [9–11] and more recently extended to include a number of other tissues and parameters [20–24], including human liver [25,26]. It was clearly revealed that all tissues [23,24] appeared to possess a GR_1 component of the glucocorticoid-specific receptor (GR) eluted in the low ionic prewash followed by a GR_4 component coeluted with blood serum transcortin in double-labelled experiments. The fact that a GR_3 moiety, in place of the GR_4 entity, was observed when synthetic steroids were substituted for natural analogues gave rise to the speculation that these two groups of molecules may activate distinct cellular components and this may explain greater potency of synthetic derivatives [22]. A GR_2 component could be observed only with corticosterone and may perform organ specific function in the liver [9–11,15,26] since it was not observed in other tissues [23]. Independent observations by others reveal at least two binding components for most steroid hormones [27]. The importance of ion selection was amply demonstrated by the fact that 10-fold greater ionic concentrations were required for elution of the same component when chloride-containing buffers were used in place of phosphate buffers and even NaCl versus KCl did not behave in a similar manner [20,21,28,29].

Certain authors have demonstrated liver receptor heterogeneity on DEAE–Sephadex A-50 columns [16] but this has been less successful in our own experience with A-50 and A-25 resins [22]. Nevertheless, receptor multiplicity was amply evident by these various techniques despite the suggestion that this may imply diversity in cell types, at least in the brain [30]. Suggestion has also been forthcoming that some of the components separated on phosphocellulose columns may bind steroid metabolites [31] and, in most studies, one or more entities exhibit

Table 1
Selected physico-chemical properties of gluco- and mineralo-steroid receptor components

Component	Steroid specificity	Tissue distribution	M phosphate elution region DEAE-52 column	Peak S–R complex conductivity (20°C) mili Siemens
GR_1	synthetic > natural	all tested	0.001 M	0.18 0.2
GR_2	corticosterone	liver only	0.02 M	5.0
GR_3	synthetic only	all tested	0.04 M	5.8
GR_4	natural only	all tested	0.06 M	9.2
MR	18-hydroxydeoxy-corticosterone	kidney	0.001 M	0.2
MR_2	aldosterone > triamcinolone	kidney, heart?	0.006 M	2.3
MR_3	deoxycortico-sterone	kidney	0.06 M	9.2
Transcortin	natural corticoids	serum tissue?	0.06 M	9.8
Albumin	all tested	plasma	0.06 M	8.9

Male, adrenalectomised rats were used throughout in all cases. In most instances, 10^{-8} M steroid concentration was used to detect the receptor component with a 30–40% organ cytosol. Elution molarities from DEAE-cellulose-52 column were brought to 20°C for determination of the specific conductivity. This was deemed more accurate than automatic temperature compensation method [5] due to less variation between experiments (unpublished). For other details see text and the corresponding reference

transcortinlike properties (see below).

A number of agents (theophylline, temperature, ions, soluble factors) activate the inert cytoplasmic receptor–steroid complex prior to its attachment with nuclear acceptor sites. The inactive form appears more acidic than the activated complex on DEAE–Sephadex columns [16] and, in an aqueous dextran–polyethylene glycol two phase system, activation is accompanied first by an increase and then a decrease in the partition coefficient of the complex [32].

Collectively, although steroid metabolism does not permit unequivocal appraisal of the nature of receptor in vivo, the polymorphic complex evident in vitro awaits further analysis via purification.

4. Mineralocorticoid receptor

The unusually labile nature of mineralocorticoid-specific receptor (MR) makes it particularly difficult to attempt any sort of physical separation. In one of the earliest attempts, chromatography on Sephadex and agarose gels revealed a single set of aldosterone–specific binding sites in toad bladder with mol. wt 100 000–200 000 [33]. Similar conclusions were arrived at using rat kidney [3].

By far the most comprehensive analysis of renal mineralo- and gluco-corticoid receptors has been carried out in the author's laboratory over the past several years. Rather than a homogeneous vector [3,4], aldosterone-specific binding proteins could be eluted as MR_1 and MR_2 units from the DEAE-cellulose-52 columns; besides kidney MR_2 was totally lacking in all other tissues hitherto tested including the liver [15,34]. MR_1 eluted in the same position as GR_1 but these could be distinguished with various mineralocorticoid agonists [35]. Thus, 18-hydroxydeoxycorticosterone was bound only to MR_1 entity whereas deoxycorticosterone was bound both to MR_1 and to a MR_3 moiety that coeluted with blood serum trans-

cortin (see below). With a synthetic steroid possessing both gluco- and mineralo- activity, 9α-fluorocortisol preferentially saturated MR in a bifunctional organ that is target for both groups of steroids [36]. Thus, only one subspecies of the complex receptor system need be saturated for physiological activity and this may form an explanation for varying potencies of different mineralocorticoid agonists. It has not yet been possible to test the behaviour of an antisteroid (spironolactone) under these conditions.

Fractionation based both on molecular weight and charge, on Sephadex A-25 columns, was less informative than DE-52 profiles due possibly to high KCl concentrations in the eluting buffer which is known to favour dissociation into monomers of comparable properties. Thus, with this procedure it was not possible to distinguish between differential binding of various mineralocorticoid agonists as described above for DE-52.

Filtration through Sephadex G-200 columns revealed a lighter peak of 67 000 daltons preceded by a heavier component of 113 000 daltons although the relative amount found was a function of steroid structure [35]. Therefore, all three aforementioned components (MR_1, MR_2, MR_3) appear to possess closely similar molecular size with possiblities of aggregation on Sephadex G-200 as frequently observed with binders for other steroids. In still other studies, only the lower molecular weight peak was observed when 0.4 M KCl was present during fractionation on Sephadex G-200 lending further support to the possibility of dimerisation under physiological conditions [37].

These differences were confirmed by actual conductivity measurements for both groups of steroids and further support the view that both GR and MR are endowed with a polymorphic, complex, heterogeneous nature that becomes evident upon physical separation and than can not be revealed by saturation characterstics alone [5].

5. Cellular 'transcortin-like' binders

Corticosteroid-binding globulin (CBG) is a protein of about 63 000 daltons capable of associating with a wide variety of steroid hormones [38]. This high capacity, low specificity carrier can be distinguished from low capacity, high affinity tissue receptor by binding characterstics. However, it is a constant source of frustration to draw a line between them on chromatographic systems due to very close molecular weights of CBG and receptor monomers and the fact that a receptor subspecies may coelute with transcortin in separations based on charge. Indeed, there has been a tendency to dismiss as transcortin all that elutes in the same position as CBG-bound steroid [39]. This is wrong.

In our laboratory, only GR_4 and MR_3 components of the gluco- and the mineralo-receptor, filled with natural tritiated hormone, coeluted with CBG-bound [^{14}C]steroid from the DE-52 column [37]. Both deoxycorticosterone (DOC) and 18-hydroxydeoxycorticosterone (18-OH-DOC) bind very avidly to CBG; yet, although DOC labelled MR_3 in the kidney, no DOC-bound radioactivity coeluted with transcortin marker when liver cytosol was used despite the fact that this organ is the site of CBG synthesis. Furthermore, 18-OH-DOC did not label any protein in the CBG region in either the hepatic or the renal cytosol [35]. Although fluorinated steroids do not generally bind to CBG [38], 9α-fluorocortisol is an exception [40]. With this potent steroid, a GR_3 entity could be labelled in the liver in keeping with GR_3 saturation by other synthetic steroids [22,37]. However, under otherwise similar conditions, 9α-fluorocortisol labelled GR_4 (and not GR_3) in rat myocardium and this coeluted with [^{14}C]corticosterone filled CBG from DE-52 column [40].

Further arguments to distinguish between transcortin and CBG-like receptors stem from reconstitution experiments. Renal cytosol-bound [^{3}H]corticosterone, in presence of excess dexamethasone to prevent corticosterone binding to glucoreceptor, could be transferred to purified nuclei whereas serum bound corticosterone did not transfer, and this was confirmed by autoradiography [3]. Furthermore, in hypotonic sucrose gradients both 8 S and 4 S peaks of bound [^{3}H]corticosterone (in presence of excess dexaethasone) were observed in renal cytosol whereas transcortin showed only a 4 S peak.

These data would argue against the idea that all cellular binders that coelute with CBG are a reflection of cytosol contamination with circulating transcortin [39]. It is however conceivable that they may represent a modified form of transcortin necessary for physiological action of selected steroid agonists. Receptor

purification is clearly required to answer this unequivocally.

6. Physiological implications

The principal difficulty in analysing the cytoplasmic receptor system stems from the labile nature of the vector protein and the fact that the carrier can be detected only after it is bound to its steroid ligand. Thus, the nature of the native receptor in cytoplasm remains entirely speculative. If the free cytosol receptor is not bound to another stabilising component of the cell, why does it remain biologically active since it is rapidly inactivated in vitro in absence of the specific ligand? To account for these and other experimental data, two sorts of post-translational modifications have been evoked. First, based on the allosteric equilibrium model of Monod et al. [41] stipulates spontaneous transformation of the low affinity form of the receptor into a high affinity form after the latter is bound to its specific ligand; to date, however, no evidence has been forthcoming to substantiate the existence of two sorts of receptors. Second, based on the 'induced fit' concept of Koshland et al. [42], predicts the existence of only one sort of cytoplasmic vector whose conformational modification is dictated by the stereospecificity of the corticoid ligand. Both models evade the basic problem of receptor stability in absence of steroid in vivo. The steroid-bound receptor is supposed to undergo an enzymatic activation (temperature-, ion- and soluble factor-dependent) leading to the appearance of positively charged groups (hence binding to phosphocellulose) while conserving the negatively charged regions that endow it with the ability to bind to DEAE prior to activation [29]. All these studies favour some sort of maturation of a more preliminary entity whose nature in situ remains unknown.

There is no general consensus on the manner in which the steroid–receptor complex of varying molecular weights penetrates the nuclear membrane which presents a barrier to macromolecules of $> 70\,000$ daltons. Once in the nucleoplasm, steroid–receptor complex is reported to interact with the acceptor. The acceptor is a concept and not an experimentally demonstrated fact although in the case of the estrogens specificity with respect to the source of nuclei is in favor of its existence. Thus, binding is possible with DNA, the acidic or the basic proteins, or even RNA polymerase [1–5]. This is further complicated by the presence of possibly only a few high affinity sites and a very large number of low affinity sites in the nucleus [43], as in the case of *lac* operon [44]. Somewhat in contrast to these is the suggestion that glucocorticoid–receptor complex binds more readily to naked DNA than to native DNA [29]. Thus, a positive control of nuclear proteins in increasing progesterone–receptor binding (and thereby response) to chick oviduct genome [45] is to be weighed against a negative control of these proteins in decreasing such response in the liver glucocorticoid model [29].

The above mentioned sequence of events was based on the assumption of a single class of cytoplasmic receptor protein endowed with one binding site. How can this be accomodated in terms of multiple molecular forms of GR and MR summarised in the preceding sections as well as similar sort of multiplicity reported for estrogen [45], androgen [46] and progesterone [47] receptors?

Thus, a second alternative would consist of different proteins each capable of binding to any one given steroid. This is difficult to visualise since an animal does not come in contact with synthetic steroids either during ontogeny or phylogeny, since cross reactivity is observed between cortisol and dexamethasone binders [31], and since redundancy in control at this level would call for several genes regulating the formation of various receptor proteins although a single mutation in receptor function is known to deplete cellular receptor for all classes of glucocorticoids in vitro [43].

Consequently, one is left with the alternative of a complex protein with different binding sites. This is not only consistent with receptor maturation mentioned above but is also supported by stereochemical considerations of steroid versus protein structure and has some experimental basis [48]. Conformation (total geometric disposition in three dimensions) is dictated by the primary structure of the protein and can not be altered in the final product. Similarly, configuration and conformation of a steroid molecule are not expected to adjust to requirements necessitated by the receptor protein. Rather, A-ring orientation may dictate potency of glucocorticoid activity in the 9α-halogenated derivatives of cortisol [49]. With mineralocorticoids, the relative in vivo potency

9α-fluorocortisol > aldosterone > DOC > 18-OH-DOC is based on substitutions in widely different positions of the steroid molecule. Since the A-ring orientation of these derivatives may be quite similar in X-ray crystallography [49], requirement for activity may reside in other, very dissimilar spatial orientations recognisable only by different, corresponding conformations of the receptor.

7. The multipolar model

Bearing in mind all these diverse experimental observations, and some hypothetical incertitudes, one can visualise the following sequence of events. The native protein is probably a very complex, uncommitted entity that may be called a Proreceptor. In presence of an active agonist of high potency and affinity, differentiation leads to an active conformation which differs from that obtained in presence of a weak synergist. Stability of the proreceptor may be assured if one were to assume that it remains bound to the ribosome until an appropriate steroid ligand were to come by for complexing. Post transcriptional modification of tRNA, HnRNA, and post translational cleavage of low molecular weight proteins from a larger precursor, are all well established regulatory mechanisms. Indeed, protease activation of a number of systems, including that of the receptor [48], is an experimentally observed fact.

Following differentiation, the Nascent Receptor would undergo a temperature-, ion- and presumably enzyme-dependent maturation into an active Holoreceptor capable of interacting with nuclear acceptor sites. Depending upon its chemical nature, an antagonist may either 'freeze' the proreceptor, thereby inhibiting interaction with active steroids in the open sites and further differentiation, or provoke an inactive conformation by actually filling the binding region thereby eliminating maturation. In the active conformation, besides the major forearm carrying the steroid for interaction with high affinity sites in DNA, the other antennae of the receptor would possess unfilled sites (hatched) with potential for association with other steroids of high affinity and for binding to chromatin in low affinity regions thereby exerting a positive [43] or a negative [29] modulation in host genome (see fig.1 for schematic representation).

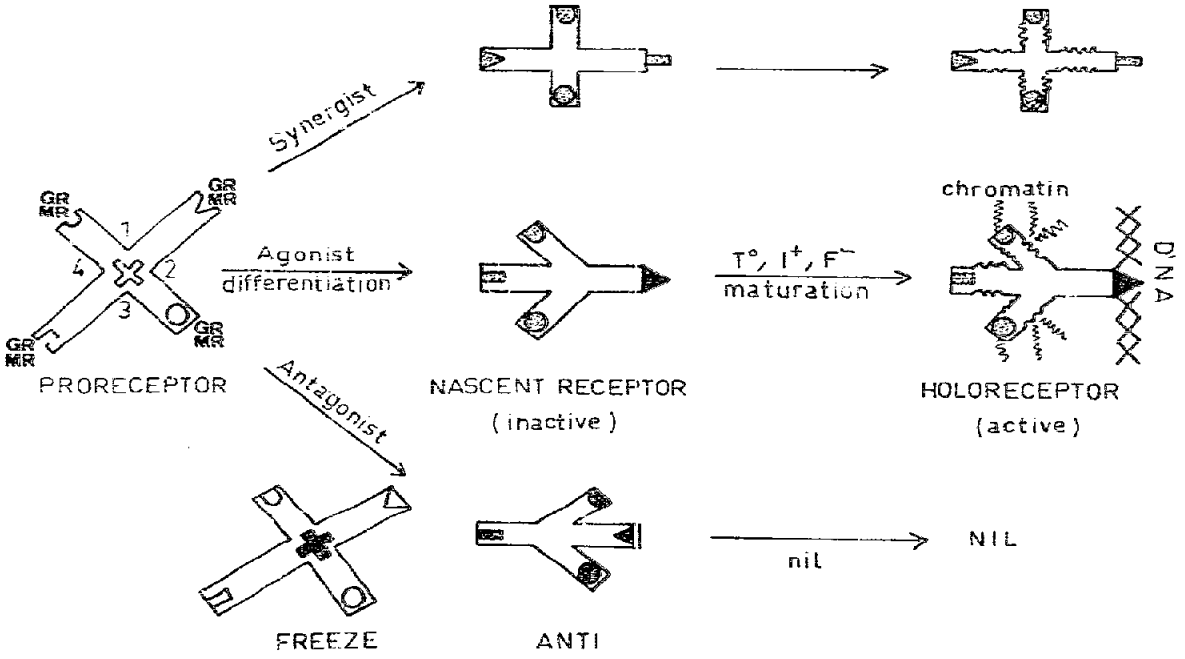

Fig.1. MULTIPOLAR MODEL OF RECEPTOR TRANSFORMATION

In this model ample provision has been made to account for the observed subspecies (GR_1, GR_2, GR_3, GR_4 or MR_1, MR_2, MR_3) that may also be construed as subunits observed in hypo- and hypertonic media. Can, in a bifunctional organ like the kidney, the pro-receptor differentiate into either GR or MR, since this would entail great economy in cellular information transfer and since most gluco- and mineralo-corticoids overlap their respective domains of action? This must await receptor purification. The possibility may also be entertained where transcortin could form a part of the complex receptor system by cleavage and repair type of mechanism known for DNA.

8. Concluding speculations

It can not be emphasized enough that a receptor can only be detected when bound to its steroid ligand and that the nature of native, uncommitted vector in cell cytoplasm is still unknown. Conceptual and technical considerations favoured propagation of receptor models where all agonists and antagonists of a steroid were supposed to interact with varying affinities to the same carrier in keeping with the view of one protein–one binding site. More detailed physico-chemical analysis revealed multiple molecular forms of the receptor for all five groups of steroids, along with preferential increases or decreases in qualitative abundance of some of these species during differentiation [19,50] and in rat liver where physiological response to glucocorticoids had been modified by prior treatment with either carbon tetrachloride or bacterial endotoxin [17,51,52]. At a purely speculative level, too, it became necessary to postulate some sort of redundancy in mechanisms required to discriminate signal from noise given the fact of multiplicity of circulating agonists as well as some cross reactivity in physiological effects (viz., gluco- versus mineralo-). Bifunctional organs (kidney) further demand consideration of control at levels not too far removed in physiological hierarchy. The Multipolar model presented here accomodates these various facets and calls for binding to both low and high affinity regions in the rather hypothetical acceptors in the nucleus. Steroids with antagonist activity are expected to provide further leads in this respect and some such work is in progress [53,54]. Finally, indulgence is asked of all those researchers who moved away from receptor characterisation as a futile exercise and who hold that the key to the specificity of steroid action lies within the nucleus. Certain risks are well worth taking to look for unity in the midst of apparent diversity.

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