Journal of Steroid Biochemistry, Vol. 11, pp. 389 to 400
Pergamon Press Ltd 1979. Printed in Great Britain

THE STRUCTURE AND REGULATORY FUNCTION(S) OF CORTISOL RECEPTOR—1: EXTRAGENOMIC EFFECTS DEPENDENT ON THE CORTISOL RECEPTOR ACTIVATION

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SUMMARY

A large body of recent data is consistent with the idea that steroids, at least sex steroid hormones, regulate gene expression in eucariotic cells via a common two-step molecular mechanism. This two-step model has acquired the status of a "dogma" and has become a generally accepted theoretical framework for experimental research being currently conducted. However, this model (dogma) does not take into consideration the immediate extragenomic effects caused by steroids in responsive tissues, nor does it offer the tentative link between extragenomic and genomic events occurring in target cells.

The results of our recent studies on cortisol specific receptor and its regulatory functions lead us to propose a new speculative model, the postulates of which are aimed at integrating both the extragenomic and genomic events occurring in target cells during the course of steroid hormone action. The key concepts of the proposed model are the following: the native cytoplasmic holoreceptor should be a multimer, consisting of several different subunits and comprising the defined "metabolic code" for various multiple cooperative metabolic functions. The "activation" of the receptor, caused by the binding of appropriate steroid hormone, results in the disaggregation of the receptor into the monomeric subunits, which play the role of regulatory proteins. The released subunits—various regulatory proteins—then exert their control at different levels of the mechanism of genetic expression, including the post-translational level. They modulate the preformed molecules and the activities of the regulatory mechanisms operative in respective target cells like for instance phosphorylation/dephosphorylation mechanism. These events result in modifications of translations on the preformed mRNA's and cell membrane transport. The resulting changes are immediate and underlie the steroid-induced extragenomic effects. The subunit(s), which binds the steroid hormone, so called "steroidophilic"—subunits, regulates the rate of transcription, i.e. selective and specific gene expression in target cells. This subunit is a key stone in the macromolecular organization of the receptor. It may be of an isoproteinic nature and may have different affinities towards other subunits thus determining the assembly and nature of other associated subunits, giving rise to the tissue specific receptor polymorphism and causing variations in the "metabolic code" of receptors. "The activation" of receptor, caused by steroids, and resulting in disaggregation of multimer into the monomeric subunits is the key event in the cell responses to steroids. It triggers, controls and links extragenomic events with gene expression. These extragenomic and genomic events are included in the "metabolic program" encoded in the molecular structure of the receptors. The steroids, as effectors trigger the receptor activation, which controls the sequential and selective metabolic events, underlying the specific physiological "responses" of steroid responsive tissues.

In this paper we will present some of our recent data to further substantiate the proposed "model" of the integral steroid hormone action in target tissues.

INTRODUCTION

One of the most challenging problems of today's molecular biology is the problem of hormonal regulation of the gene activity in eucariotic cells. Precise molecular mechanism(s) of steroid hormone–genome interaction are not elucidated yet. However, an ever-increasing body of recent data agrees that various steroids, at least sex steroid hormones, regulate gene expression in eucariotic cells via a common two-step molecular mechanism [1–6].

This two-step model has already acquired the status of a "dogma" and has become a generally accepted theoretical framework for experimental research which is currently being conducted. Although the postulated sequence of events may, probably, be the primary mechanisms by which the

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steroid regulates the function(s) of the genome, nevertheless, the steroid hormones have been reported to trigger in target cells a series of effects within time intervals much shorter than those necessary for the hormone-induced modulation of gene expression. These immediate non-genetic effects are termed extragenomic effects. The present "dogma" does not take these extragenomic effects into consideration. Thus, for example, cortisol induces immediate phosphorylation of the liver ribosomal and nuclear proteins [7–9]. In addition steroid hormones affect the changes in cell permeability [10], in polyribosomal distribution [11] in cyclic 3'-5' adenosin monophosphate levels [12], in histamine release [13], etc. A satisfactory hypothesis to account for these immediate short term acute extragenomic effects, mediated by steroids, has not, as yet, been formulated.

The results of our recent studies on cortisol specific receptor and its regulatory functions in anabolic (liver) cells and catabolic target (thymus) cells lead us to propose a new model-working hypothesis—the postulates of which are aimed at integrating both the immediate (acute) extragenomic and late genomic events, occurring in target cells during the full course of steroid hormone action. We propose that the native cytoplasmic holoreceptor should be a multimer consisting of several regulatory proteins (subunits) and comprising the "metabolic code" for multiple cooperative functions. The "activation" of receptor, caused by the binding of specific steroid, results in the disaggregation of the receptor into the monomeric subunits—regulatory proteins. It plays a key role in the regulation of extragenomic and genomic events and in the changes occurring in the target cells. The released subunits—various regulatory proteins—exert thereafter their regulatory role at transcriptional, post-transcriptional, translational and post-translational levels. Their modifying action on the molecular mechanisms, operative in target cells, underlies the acute (immediate) extragenomic effects, caused by the hormones in target cells. The subunit(s) which binds the steroid hormone or the so called "steroidophilic" subunit(s)—regulate(s) the rate of transciption—gene expression, i.e. the rate of the *de novo* synthesis of a few specific proteins. Thus, the "activation" of specific cytoplasmic receptor by steroids is a crucial event in the cell "responses" to steroids. It triggers and stimulates both extragenomic and genomic modifications, being comprised in the program of receptors "metabolic code". The primary role of steroids should, therefore, be to "activate" the specific native receptor, that is to "program" sequential and selective metabolic functions underlying the specific physiological "responses" of target cells and responsive tissues.

The basic ideas of the proposed unifying hypothesis have already been described, elsewhere [5, 6, 14]. In this paper we will present some of our recent efforts to further substantiate our proposed "model" of steroid hormone action.

EXPERIMENTAL

Animals. Male Mill Hill hooded rats weighing 200–250 g were used. The animals were given food and water *ad lib.*, and left to starve for 14–18 h before sacrifice by cervical decapitation.

Materials. [1,2,6,7,^{3}H (N)]-Cortisol (80.6 Ci/mmol) and [1,2,4,-^{3}H (N)]-dexamethasone (20.9 Ci/mmol) were purchased from New England Nuclear Corporation. [^{32}P]-Orthophosphate, carrier free (3.16 mCi/ml) was obtained from the Institute for Nuclear Sciences "B. Kidrič"-Vinča. All other chemicals were p.a.

Injection of unlabelled hormone and [^{32}P]-orthophosphate. The animals were injected i.p. with 5 mg/100 g or 2.5 mg/100 g body wt. of unlabelled hormone (as presented in relevant Figures) and 1 mCi of ^{32}P per 100 g body wt. ^{32}P was given as NaH_2PO_4 salt. Control animals received isotope and 0.9% NaCl.

Preparation of cytosol receptor(s) and nuclei. Preparation of cytosol, the partial purification of receptor protein(s), or receptor-hormone complexes by ammonium sulphate precipitation, isolation of nuclei by 2.2 M sucrose were performed as previously described [6, 15]. Partially purified phosphorylated receptor-hormone complex was analysed using agarose gel electrophoresis [6]. The incubation of cytosol with labelled hormones (at final concentration of 3×10^{-8} M), the activation by heat of partially purified complexes, and the separation of different receptor forms before and after activation, using DEAE-cellulose or DEAE-Sephadex ion exchange chromatography, were carried out as described earlier [6, 15, 16].

Binding assay. To determine the amount of bound hormone and specific bound hormone Dextrane-coated charcoal assay was used [17] before the application of all other methods.

Phosphorylation of ribosomal proteins. The post-mitochondrial supernatant, ribosomes and ribosomes subunits were prepared as previously described [18]. Mg-acetate up to 0.1 M and 2 vol. of acetic acid glacial were added to ribosomal solution (25–30 A_{260} units/ml) and then proteins were extracted [19].

In order to determine the level of phosphorylation of ribosomal proteins the filter strips carrying the radioactive ribosomes of ribosomal proteins were processed as follows: paper strips were first treated by 10% TCA at 0°C for 30 min in order to extract acid soluble compounds. Phospholipids were then extracted by chloroform–methanol (2:1, v/v) (30–60 min). RNA was hydrolysed by 5% TCA at 95°C for 15 min. All residual ^{32}P was essentially contained in phosphoproteins. Radioactivity was determined in a Nuclear Chicago liquid scintillation counter.

Determination of protein. Protein was determined by the method of Lowry *et al.*[20] with bovine serum albumin as standard.

RESULTS AND DISCUSSION

1. *Structure and activation of cortisol receptor*

Target cells and tissues preferentially retain the respective steroid hormone due to specific cytoplasmic

receptor. The key stone of the hypothesis which we propose is the molecular structure and organization of cortisol native holoreceptor and its activation. The primary function of steroids, as allosteric effectors, is to facilitate the "activation" of specific receptor.

It should be emphasized that the true nature and organization of the native cytoplasmic steroid holoreceptor is still unknown and is entirely speculative. Consequently, when we are talking about the receptor we are simply thinking of the complex formed with "steroidophilic" subunits of holoreceptor. Bearing in mind diverse experimental data and on the basis of the variety of experimental evidence from our laboratory it appears logical to us to propose that the native steroid holoreceptor is a specific multimacromolecular system, which consists of several different subunits—various regulatory proteins. The subunit of receptor which binds the respective steroid i.e. "steroidophilic subunit", seems to be of isoproteinic nature, and acts as the key stone in the organization of native receptor system. This "steroidophilic" subunit, being isoprotein, may aggregate with several different subunits, giving rise to different macroloecular forms of native functional receptor. This would imply that the structure of a given steroid receptor may vary from tissue to tissue and may be "encoded" with different metabolic programmes (as for instance cortisol receptors in the liver cells and in lymphocytes). The proposed model offers plausible explanations for the heterogeneity, and tissue specific polymorphism of steroid receptors. Consequently the activation of a particular receptor, i.e. disaggregation of macromolecular holoreceptor system in various tissues may cause different metabolic events which underlie the activation of different "batteries" of genes. The function of steroid hormones is to "activate" the receptor regulatory system, but the patterns of induced metabolic changes are dependent on the structure of the receptor.

How far may the proposed model be substantiated by experimental data? Is native receptor a multimeric molecule?

Our earlier data on the receptor–cortisol complex, isolated from the rat liver cell cytoplasm, have been presented elsewhere [5, 6, 15].

In an attempt to get a better insight into the structure of cortisol and dexamethasone receptors, we studied thermal activation of these receptor systems.

The cytoplasmic cortisol receptor can be resolved by ion exchange chromatography into three components (fractions) (Fig. 1A). All of them bind cortisol but their specific capacity to bind [^{3}H]-cortisol at 25 and 4°C varies (Fig. 1B). The nonactivated complex formed at 4°C was found to elute as one peak immediately after void volume (Fig. 2A). However, the [^{3}H]-cortisol complex activation at 25°C, has been resolved into two components (subunits) (Fig. 2B).

In order to test the ability of cortisol–receptor complexes, formed under different temperature conditions, to interact with chromatin acceptor binding sites we followed their uptake by isolated liver cell nuclei. The uptake was markedly higher if nuclei were incubated with activated (25°C) receptor complex than with that formed at 4°C. When inactivated complexes, formed at 4°C, were shifted to 25°C the amounts of [^{3}H]-cor-

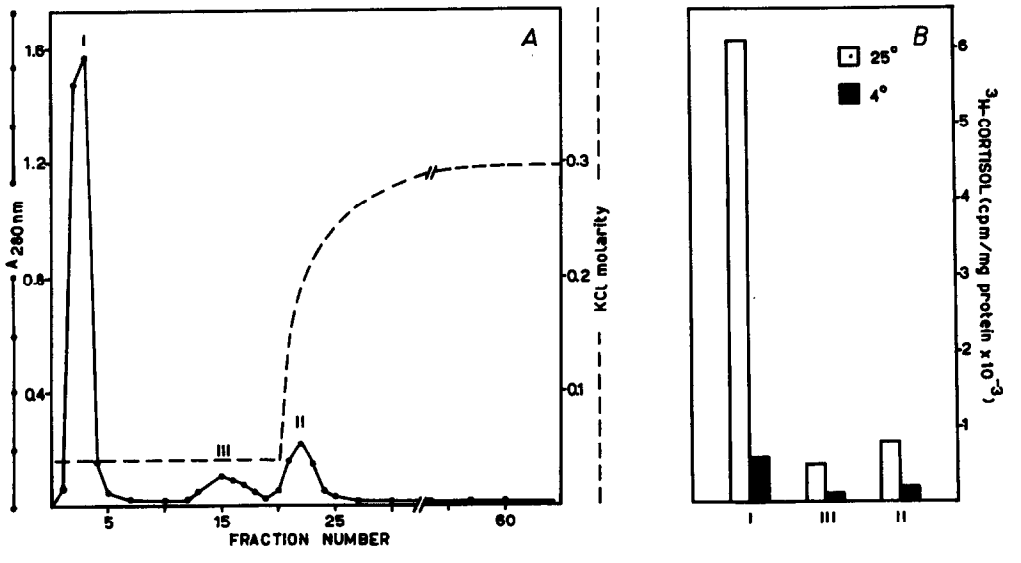

Fig. 1. The fractionation of receptor components from the rat liver cytosol. (A) Ion exchange chromatography of precipitated cytosol fraction. The liver cytosol was precipitated at 20–35% of ammonium sulphate saturation and then analysed by means of DEAE-cellulose chromatography as described in Experimental. (B) [^{3}H]-Cortisol binding with receptor components. The fractions (designed as I, II, III at previous diagram) obtained after ion exchange chromatography of precipitated liver cytosol were incubated with 30 nM [^{3}H]-cortisol at 25°C for 30 min, or at 4°C for 90 min, and after these time intervals the specifically bound hormone was determined by Dextran–charcoal method.

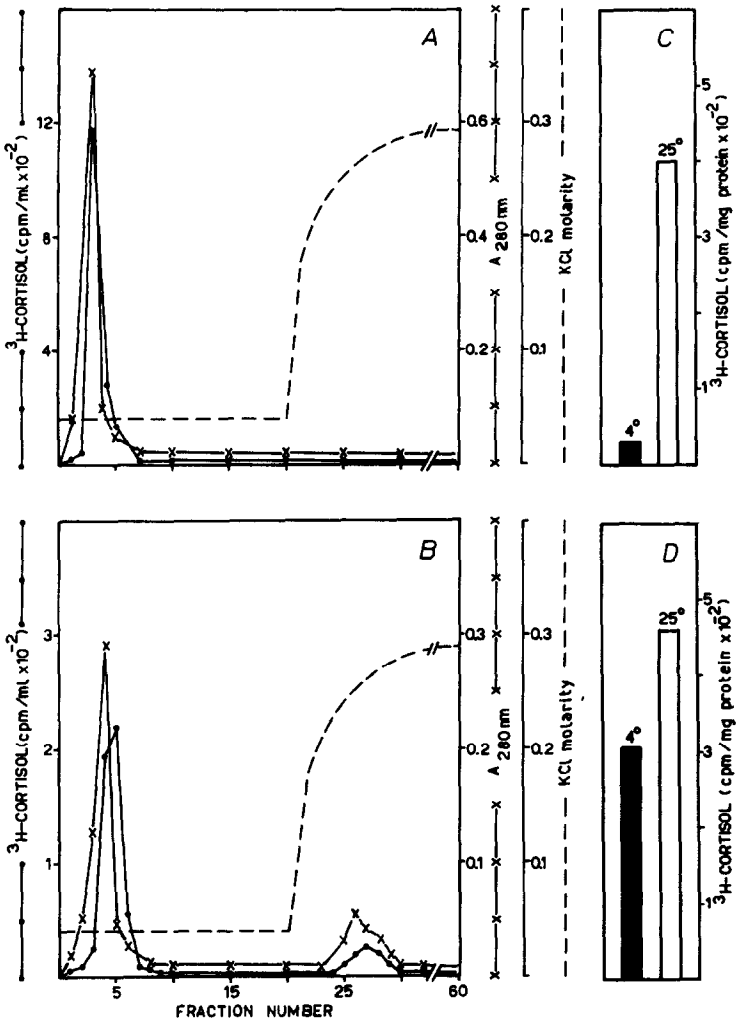

Fig. 2. The activation of [^{3}H]-cortisol receptor complex(es). Chromatography of unactivated (A) and activated (B) forms of [^{3}H]-cortisol-receptor complex. The first component obtained after chromatography of precipitated cytosol was incubated with 30 nM [^{3}H]-cortisol at 4°C for 90 min. Nonspecific bound and free radioactivity was eliminated by Dextran–charcoal treatment and it was again submitted to chromatography (A). The protein fractions with bound [^{3}H]-cortisol were collected and following activation (heating them up to 25°C) they were subjected to rechromatography on column of DEAE-cellulose (B). Nuclear uptake of unactivated (C) and activated (D) [^{3}H]-cortisol-receptor component. Liver nuclei were incubated with unactivated (C) and activated (D) forms of [^{3}H]-cortisol-receptor component obtained after chromatography as described above. The incubations were done at 4 or 25°C for 30 min. After this time interval, the specific uptake was expressed as c.p.m./mg protein in nuclear protein fraction.

tisol receptor complex taken up by nuclei increased markedly if they were incubated either at low (4°C), or higher (25°C) temperature (Fig. 2C,D).

This finding is in correlation with the results concerning the activation of dexamethasone receptors (Fig. 3). It is shown that only the activated form of the receptor, separated from nonactivated complex by DEAE-Sephadex ion exchange chromatography, could bind to DNA-cellulose.

These data seem to support the basic concepts of our "model". Namely, our data suggest that the native cortisol holoreceptor is, very likely, a multimeric labile macromolecular system which disaggregates on activation. Thus, so far we have not established the exact number of components of the cortisol specific cytoplasmic holoreceptor, but under some experimental conditions 3–4 components can be separated by temperature activation. These components are, we believe, the subunits of the receptor system. All of them have the capacity to bind cortisol, but only one, called "steroidophilic" is translocated to the nuclei upon activation. The temperature activation increases its capacity to interact with nuclear proteins. We can not yet answer directly the question of what would be the role of other subunits, but we have, at present, the feeling that they are involved in the control of immediate extragenomic events and late gene expression.

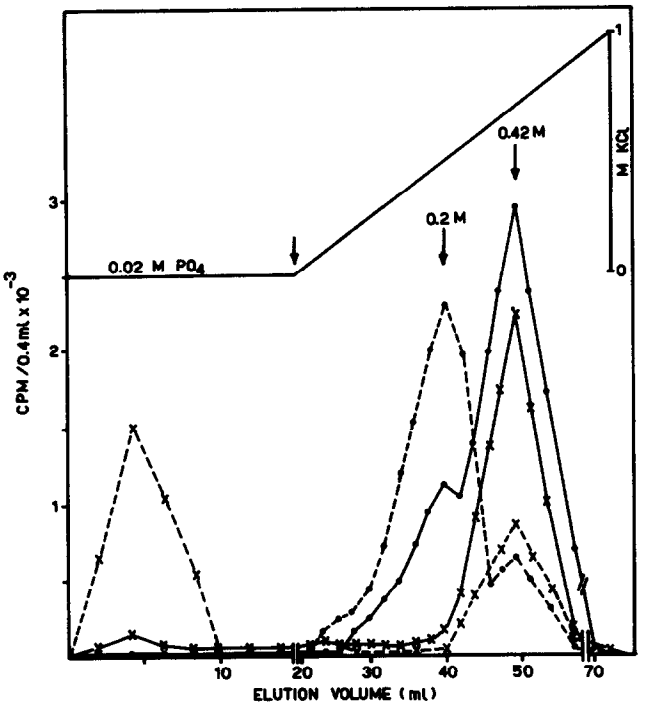

Fig. 3. DEAE–Sephadex chromatography of non activated and activated [^{3}H]-dexamethasone-receptor complex from the rat liver and kidney cytosols. Cytosols were labelled with 30 or 50 nm [^{3}H]-dexamethasone respectively and chromatographed on minicolumns using linear KCl gradient elution system. ●——● non heated control—liver; ●---● heat activated—liver; ×——× non heated control—kidney; ×---× heat activated—kidney.

Our data relative to the activation of the liver and kidney [^{3}H]-dexamethasone receptor (Fig. 3), showing that the activation, the level of disaggregation is different in the examined tissues, and, furthermore, that some factors present in the kidney cytosol affect the activation of the liver complex, suggest that the "activation" of receptor is more than a simple disaggregation of subunits [16]. The results we presented are also in agreement with the data concerning the receptors of sex steroids [21, 22].

Activation of cortisol receptor and its phosphorylation

It is still very premature to speculate on the mechanism of the receptor "activation" *in vivo* since we still do not know enough about the structure, organization and composition of the native holoreceptor. However, one could expect to observe the "autocatalytic" regulation of the "activation" founded on the basic principles of phosphorylation dephosphorylation mechanism. Namely, one of the released subunits might catalitycally regulate the modifications of other receptor subunits. In an attempt to test this plausibility we have studied the effect of cortisol on the phosphorylation of its receptor. After administration of cortisol the phosphorylation of bulk cytoplasmic proteins and receptor proteins purified by ammonium sulfate precipitation and agarose gel electrophoresis was followed at different time intervals. The results obtained strongly suggest that the maximum phosphorylation of the receptor protein occurs in the first 10 min. The marked ^{32}P incorporation into receptor protein was demonstrated by agarose gel electrophoresis (Fig. 4). This finding suggests that the phosphorylation of holoreceptor might be a step in the receptor "activation" process. However, our data indicate that the binding of the phosphorylated "steroidophylic" receptor complex to nuclear proteins, extractable by 0.3 M NaCl, is more efficient than that of the receptor prepared from untreated animals. The results of these experiments are given in Fig. 5. The nuclear uptake of hormone-phosphorylated receptor complexes was three times higher than that of the receptor prepared from the liver of the controls.

Our data are consistent with the observations that liver glucocoricold receptor can be inactivated by dephosphorylation and that only the phosphorylated form of receptor is capable of binding steroid [23]. Therefore, at present we can not state that the "activation" is simply an intrinsic property of the receptor regulated only by the hormone binding. It might also be an "autocontrolled" catalytic process.

2. *The role of cortisol receptor in the mediation of cortisol dependent extragenomic effects*

The extragenomic effects at the translational level

Our model postulates that the "activation", i.e. disaggregation of specific cytoplasmic receptor into the monomeric subunits, is a key event in the hormonal regulation of metabolic processes in the target cells.

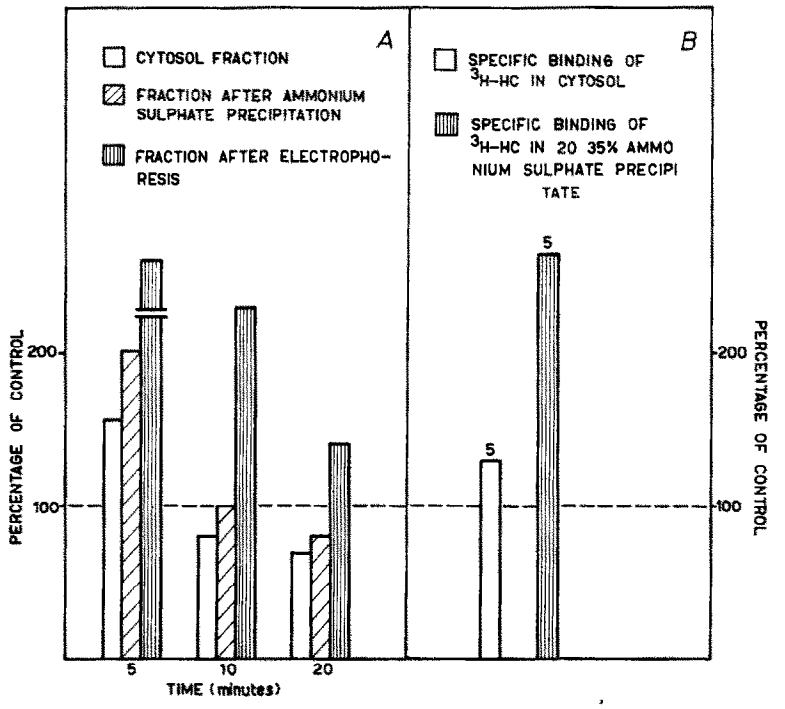

Fig. 4. Time course of endogenous phosohorylation of total cytosol proteins and cortisol-receptor protein purified by ammonium sulphate precipitation or agarose gel electrophoresis. (A) The animals were injected with cortisol and [^{32}P]-orthophosphate (2.5 mg/100 g and 1 mCi/100 g body wt.) simultaneously and sacrificed at the indicated times. The control animals were treated with [^{32}P]-orthophosphate alone. The aliquots of cytosol were precipitated with solid ammonium sulphate, or subjected to agarose gel electrophoresis. (B) The standard assay system for studying the effect of ATP on phosphorylation of receptor and specific cortisol binding capacity included: 0.5 mM Zn-acetate, 3 μM ATP and 3×10^{-8} M [^{3}H]-cortisol (or 3×10^{-5} M cortisol). The mixtures were incubated for 5 min at 25°C. The aliquots of cytosol were precipitated with 20–35% ammonium sulphate. The controls were incubated in the absence of ATP.

We have assumed that the "activation" of receptor in anabolic target cells may result in releasing of free subunits some of which may have protein kinase activity, or may modulate the activity of preformed protein kinases, which is likely to be involved in the phosphorylation of polysomes and ribosomes. In an attempt to test this plausibility we followed the cortisol dependent phosphorylation of polysomes and ribosomes in the liver cells.

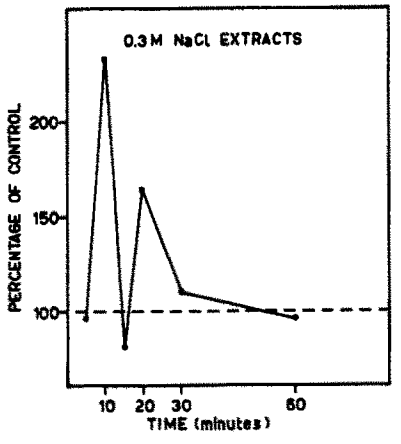

Fig. 5. Nuclear binding of phosphorylated receptor-cortisol complex. The isolated liver nuclei were incubated at the shown time intervals with cortisol-receptor complex prepared from the liver slices incubated in the presence of cortisol and [^{32}P]-orthophosphate (treated), or [^{32}P]-orthophosphate alone (control). Incubation procedure was performed at 25°C. Radioactivity bound to nuclei was measured in 0.3 M NaCl extracts.

Cortisol dependent phosphorylation of the rat liver polysomes and ribosomes

The phosphorylation of translational proteins and/or specific ribosomal proteins has been suggested to be implicated in the control of the translation [24]. Having this in mind, we studied the immediate effects of the cortisol injected on the level of phosphorylation of ribosomes and on the rate and the pattern of translation.

After cortisol administration the level of intracellular phosphorylation of ribosomal proteins was followed by [^{32}P]-orthophosphate incorporation into the ribosomes of the rat liver cells. This analysis revealed the increased level of phosphorylation of the liver cells ribosomes 5 min after cortisol administration. The highest level of phosphorylation of ribosomes occurs 10 min after cortisol administration (Table 1). At that time interval the labelling of the liver ribosomal proteins increased by 1.5–2 times as compared with the control ribosomes (Table 2). The ribosomes were dissociated into subunits. The small (40 S) and

Table 1. The changes in the level of phosphorylation of the rat liver ribosoma proteins after cortisol treatment

Cortisol treatment (min)	Labelling time (min)	[^{32}P]-Radioactivity of ribosomal proteins percent of control
0	10	100
10	10	153
20	10	119
40	10	110

Rats were injected i.p. with 4 mg/200 g body wt. of cortisol and sacrificed after 10, 20 and 40 min, respectively. Ten minutes before sacrificing animals were injected with 3 mCi/200 g body wt. of [^{32}P]-orthophosphoric acid. Ribosomes were prepared and the radioactivity determined as described previously. The radioactivity values are given in percentages of control value taken as 100%.

large (60 S) subunits were separated in sucrose density gradients, containing 0.3 M KCl. The level of phosphorylation of proteins of both subunits was increased markedly 10 min after hormone administration (Table 3). The overall phosphorylation of proteins of 60 S subunits was 30–40% higher than that of proteins of 40 S subunits. Two dimensional acrilamid gel electrophoresis revealed that the phosphorylation of several proteins of large subunits was dependent on cortisol, i.e. very likely on the activation of cortisol specific receptor. Identification of these large subunit proteins is now in progress. Among proteins of small 40 S subunits, S-6 protein was labelled, but there was a difference in its labelling after cortisol administration. These findings suggest that cortisol, very likely through the "activation" of the receptor, regulates the level and the pattern of phosphorylation of the ribosomal proteins.

What is the physiological meaning of this cortisol mediated phosphorylation of ribosomes?

There is no precise answer to that question. However, the changes in the level of ribosomal proteins phosphorylation, occurring within the cells after the administration of proteohormones or cAMP [25–27], after the partial hepatectomy [28] and after the viral infection of HeLa cells [29] suggest that the phosphorylation of ribosomes may be involved in the control of ribosomal functions *in vivo*. The number of modified ribosomal proteins phosphorylated *in vivo* is not yet precisely established.

The most important remaining problem concerns therefore the role of ribosomal protein phosphorylation. We believe indeed that the phosphorylation of ribosomal proteins must have essential function(s) because, as our data suggest, this phosphorylation in anabolic target cells is subject to hormonal control and is likely dependent on the activation of cortisol specific receptor. However, very preliminary data from our laboratory indicate that in cortisol sensitive thymocytes catabolic target cells there is no increase but rather a decrease in the level of phosphorylation of ribosomes. The kinetics of phosphorylation of the brain cells polysomes after cortisol injections differs also from that of the liver ribosomes (Selma Kanazir personal communication). Therefore, it is tempting to speculate that short term increase of the phosphorylation level of some ribosomal proteins may rather be involved in changing the pattern of translation, mediated by the selection of specific mRNAs for translation, than in changing the synthesis of bulk proteins.

In order to probe this assumption we followed protein synthesizing capacity and pattern of the liver cells, as well as the translation capacity of polysomes after cortisol injection with time intervals shorter than those needed for the gene expression, i.e. translation of newly synthesized mRNAs.

Effects of cortisol on the pattern and the rate of translation in the liver cells

It seems likely that the activation of steroid receptor may control the specificity and efficiency of trans-

Table 2. Incorporation of radioactivity of [^{32}P]-orthophosphoric acid into the protein of ribosomes in normal and cortisol treated rats

Source of the liver ribosomes	Labelling (min)	[^{32}P]-Radioactivity in ribosomal particles (c.p.m./μg of protein)
Control animals	5	1.8
	10	3.0
Cortisol treated animals	5	5.1
	10	9.2

Rats were injected i.p. with 10 mg/200 g body wt. of cortisol and 2 mCi/200 g body wt. of [^{32}P]-orthophosphoric acid. The animals were sacrificed 5 and 10 min later. Radioactivity was determined as in the legend to Table 1.

Table 3. Effect of cortisol on the incorporation of the radioactivity of [^{32}P]-orthophosphoric acid into rat liver ribosomal subunits

Treatment	[^{32}P]-Radioactivity in ribosomal subunits (c.p.m./μg of protein)			
	40 S	% of control	60 S	% of control
Normal	23.6	100	10.3	100
Cortisol treated	34.6	147	18.6	181

Rats were injected with 10 mg/200 g body wt. of cortisol and 2 mCi of [^{32}P]-orthophosphoric acid. The animals were sacrificed 10 min later. The ribosomes were isolated from the livers and the nascent polypeptides were released by treatment with 0.1 mM puromycin; treated ribosomes were separated into subunits by sucrose gradient centrifugation. The subunit proteins were extracted by acetic acid procedure and the radioactivity was determined as in the legend to Table 1.

lation of some particular preformed mRNAs in the target cells. This idea seems to be also supported by data on the sex steroid hormones [30].

To test this assumption we followed *in vivo* the rate of incorporation of ^{14}C-labelled amino acid into the liver cytosol proteins (solubile proteins) within 120 min of cortisol administration. Within that time interval no increase in the labelling of the total (bulk) rat liver soluble proteins was observed, but the increased rate of synthesis of cortisol receptor—specific proteins—was revealed. A significant increase of the labelling of cortisol specific receptor was observed 10 min after cortisol administration. The rate of incorporation of the labelled amino acid into the receptor molecules was four to five times higher than in the controls (Fig. 6).

Pretreatment of animals with actinomycine D 30 min before administration of cortisol and ^{14}C-labelled amino acids did not affect immediate (10 min peak) receptor synthesis. This indicates that early synthesis of specific receptor proteins does not require *de novo* synthesis of receptor specific mRNA, i.e. transcription. The early 10 min peak of cortisol receptor synthesis, unaffected by actinomycine D, but abolished by cycloheximide, can be attributed to the translation occurring on the preformed specific mRNAs and may be correlated to the activation of cortisol specific receptor [6]. This finding may indicate that the "activation" of receptor may be the event which is involved in the selective translation of some preformed specific mRNAs. This indicates as well that cortisol regulates and controls the rate of synthesis of its own receptor, i.e. the specificity and efficiency of translation of some particular mRNAs. This accelerated selective translation, observed 10 min after hormone injection, can not be attributed to the increased rate of transcription, but rather to modifications of some other mechanism(s) controlling the translation such as phosphorylation, and/or acetylation of ribosomes, and/or translation, initiation, elongation or termination factors. It is of interest to note that our idea is supported also by findings that cortisol activates the liver protein chain elongation processes within very short time intervals, such as 5 min. Under these conditions hormone causes a preferential stimulatory effect on the labelling of nascent polypeptide on free polysomes [31].

Our results are also in good agreement with the data relevant to the mode of action of sex steroids. Namely, oestrogen induces in the rat uterine cytosol a specific protein called "induced protein". No specific function has been assigned to this protein.

Numerous androgen-inducible proteins have been

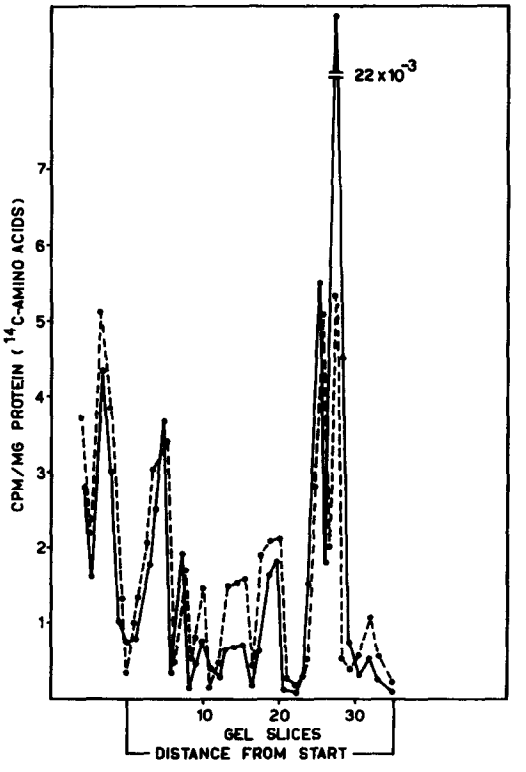

Fig. 6. The electrophoretic distribution of the liver soluble proteins. The rats were injected simultaneously with ^{14}C amino acids (50 μCi/100 g body wt.) and cortisol (2.5 mg/100 g body wt.). The rats were sacrificed and cytosol fractions from the livers prepared 10 min after cortisol and labelled amino acid injection (treated), or 10 min following the administration of labelled amino acids (control). The aliquots of cytosol were subjected to agarose gel electrophoresis and soluble proteins were separated (●---● control; ●——● treated).

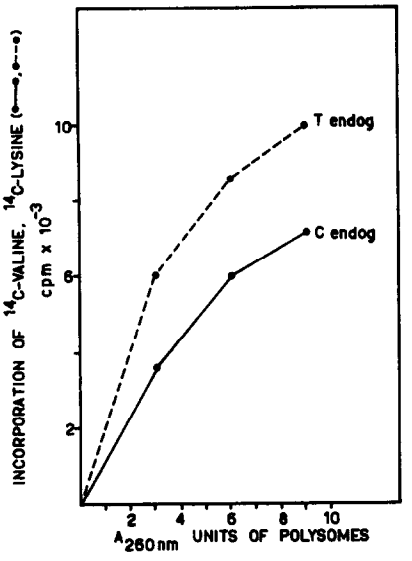

Fig. 7. Effect of cortisol *in vivo* on incorporation of amino acids *in vitro* Animals were sacrified 10 min after cortisol injection. Reaction mixture contained in volume of μl: various amounts of polysomes fraction as indicated, 50 mM Tris, pH 7.6, 80 mM NH_4Cl, 6 mM Mg-acetate, 6 mM mercaptoethanol, 2.5 mM ATP, 0.5 mM GTP, 10 mM PEP, 10 μg PEP kinase, a mixture of 18 amino acids lacking valine and lysine (25 mM each) and 1.0 μCi of labelled amino acids for endogenous incorporation. Samples were incubated at 37°C for 30 min. The radioactivity of heat stable acid precipitable material was determined. Incorporation with different polysomes concentration: (●——●) endogenous incorporation of valine and lysine in controls, and (●---●) in cortisol-treated animals.

identified. However, castrated rats of either sex do not respond to androgen treatment by the induction of these protein(s). Thus, the failure to respond is correlated to the absence of androgen receptor in the livers of such animals [32].

All the above mentioned data suggest that the activation of steroid receptor very likely increases the selectively of translation on some particular preformed mRNAs. Whether the released subunits act directly on translational machinery remains to be elucidated.

Cortisol and ribosome translation capacity

In an attempt to study immediate extragenomic effects dependent on cortisol, we studied the translation capacity of polyribosomes prepared from the rat liver (anabolic target) cells 10 min after cortisol administration. Within this time interval, cortisol induced *de novo* synthesized mRNAs were not yet transported from the nuclei to the cytoplasm [33, 34]. Consequently, any change in polyribosomal translation capacity can be attributed to the cortisol dependent modifications of ribosomes and/or translational factors. For that purpose the animals were sacrificed 10 min after cortisol administration. The liver polyribosomes were prepared from both treated and control animals and their translation capacity was tested in an *in vitro* system. The rate of incorporation of [^{14}C]-valine and [^{14}C]-lysine in polysomes has been followed. The samples were incubated for 30 min at 37°C and the radioactivity of heat stable acid precipitable material was determined. The polyribosomes, prepared 10 min after cortisol administration from the livers of treated animals, showed an increased capacity to stimulate the synthesis, i.e. the incorporation of labelled amino acids was directed by endogenous mRNAs (Fig. 7).

In the same *in vitro* system an increased capacity of the rat liver ribosomes and S-100 fraction to translate poly-U was observed [6]. This finding suggests that increase of translational capacity can be attributed to the immediate chemical modifications of ribosomal proteins and/or elongation factors such as transferases I and II, involved in the elongation of the peptide chain.

Our results relevant to the cortisol induced increase of translational capacity of polyribosomes are in good agreement with the data from other laboratories. Thus, the protein synthesizing activity of isolated rat prostatic ribosomes was reduced by castration, whereas testosterone treatment restored this activity [35]. Furthermore, mRNA fractions from testosterone-treated castrated rats were much more active, than that from castrated controls, in directing protein synthesis in a cell free translational system derived from Krebs Ascites tumour cells [36]. The capacity of Xenopus liver ribosomes to incorporate, in cell free systems, amino acids into protein was found to be increased by oestrogen administred *in vivo* [37, 38]. Furthermore, cortisol administration has been found to increase the clustering of ribosomes per mRNA strand and to stimulate the labelling of nascent polypeptides on free polysomes [31, 39]. The formation of heavier polysomes, induced by oestradiol in the chick oviduct, was also reported [40].

Our data, therefore, indicate that the translation capacity of ribosomes is markedly increased very early after cortisol administration. It seems to be a general phenomenon valid for all steroid hormones [6, 31, 37–40].

3. Possible link between steroid-cortisol-receptor activation and phosphoprotein kinases activity

It is evident that the steroid hormones are involved in the phosphorylation of various pre-existing proteins in respective target cells and tissues, but the mechanis(s) and the involvement of cyclic nucleotides in the action of steroid hormones is less clear. We propose, within the frame of our "model", a very tentative mechanism aimed to correlate the level of phosphorylation of preformed proteins with steroid receptor activation. The activation of steroid receptors and phosphorylation seem to be a time-linked series of events. Therefore, we propose that cortisol (steroid) receptor may contain as subunits either protein phosphokinase (or phosphokinase modifying protein) or phosphatase depending on its tissue speci-

ficity. This view is very strongly supported by the observation that a rapid decline of type I protein kinaze activity parallels the postcastration decay of receptors [41]. Consequently, the activation of receptor would control either the intracellular concentration, i.e. the synthesis and/or activity of preformed phosphoprotein kinases, or phosphatases varying with respect to target tissues. Our concept is that steroids are contributing to the maintenance of kinase or phosphatase pools in respective anabolic or catabolic target tissues. The phosphoprotein kinases and phosphatases are involved in the regulation of adenilcyclase activity. Consequently, steroids may also indirectly regulate intracellular cAMP. For instance, it is shown that the absolute level of cAMP and its rate of biosynthesis are dependent on the gonadal status of animal [42]. The adenilate cyclase activity, cAMP levels and polyamine levels decrease in the ventral prostate of the rat after anti-androgen administration. The action of cAMP is impaired in the absence of glucocorticoids [43].

However, there are data indicating that steroid hormones control the phosphorylation of the steroid and cAMP regulated phosphoprotein (SCARP) in target cells. This protein has been tentatively identified as the regulatory subunit of protein kinase [44, 45]. The present data suggest that steroids regulate either the amount of SCARP or its ability to become phosphorylated. Cyclic AMP controls endogenous phosphorylation of SCARP. Certain considerations suggest that SCARP may be the regulatory subunit of cAMP-dependent protein kinase and that it may undergo autophosphorylation by the catalytic subunit of the enzyme. The function(s) of SCARP is not identified yet but it might be involved in the regulation of protein synthesis, especially at the level of translation. We assume that the activation of steroid receptors might control its synthesis on preformed mRNA, whereas the SCARP might be involved in the selective translation of some preformed mRNAs. The function of SCARP would be regulated by phosphorylation and dephosphorylation which are mediated by cAMP and/or cAMP-independent kinases and by specific phosphatase [46]. Therefore, the SCARP kinase/SCARP phosphatase system might have a wide spread and a significant role in the regulation of gene expression at the translational level, i.e. in the selection of mRNAs to be translated. This system might be a direct link in the primary chain of molecular events by which steroids elicit their extragenomic and genomic regulation. In addition, steroid control of cell permeability may be related to their involvement in the regulation of membrane proteins phosphorylations. It is perhaps too early to speculate, but one might assume that one of the steroid receptor subunits might be either "catalytic subunit" of cAMP-dependent or independent phosphoprotein kinases specific of SCARP, or SCARP specific phosphatase. In view of our unifying hypothesis it would seem important to determine whether steroid holoreceptor system contains as subunits: catalytic of regulatory subunits of specific cAMP dependent or independent phosphoprotein kinases or specific phosphatase. The composition of holoreceptor with respect to these subunits may vary from tissue to tissue. This may elucidate the tissue specific polymorphism of the steroid receptors, the variations of their functions in anabolic and catabolic target tissues, as well as the well documented sinergism between the steroid hormone action and polypeptide hormones, the effects of which are cAMP mediated.

CONCLUSIONS

In an attempt to integrate both extragenomic and genomic effects dependent on and controlled by steroid hormones we propose a speculative "model" relative to the structure, the molecular organization and the function of steroid receptor.

Speculations relative to the structural organization of receptor

All lines of evidence from our and other laboratories seem to support the view that cytoplasmic steroid hormones receptors have a much more complex structure and organization than we thought until now. Our knowledge of the structure of cytoplasmic steroid receptors or organization is still very poor.

Having available data in mind it appears logical to us to propose a speculative model on receptor structural and molecular organization. The model postulates that a native steroid holoreceptor is a multimer, aggregate of various subunits—macromolecular system—comprising well defined "metabolic code", i.e. metabolic programmes encoded into its structure. The binding of steroid hormones to the respective cytoplasmic receptor systems initiates the "activation" of receptor which results in the disaggregation of the receptor system back to the original monomeric subunits. The key stone of molecular organization of the native receptor system is the "steroidophilic" subunit, which exhibits an extremely high affinity and specificity toward steroids and other subunits. The "activation" of native receptor increases the affinity of this subunit for nuclei, i.e. acceptor sites and stimulates the translocation of complex "steroidophilic" subunit—hormone to nuclei. The complex, i.e. "steroidophilic" subunit(s) seems to be a regulatory protein controlling the rate of transcription, i.e. the genes expression and causing of genomic effects. This subunit is a key subunit determining the pattern of assembly (aggregation) of other subunits in the native receptor system(s).

The tissue specificity of the receptor, as well as the specificity towards the steroid, may be explained by isoproteinic nature of steroidophilic subunits (isoproteins). This implicates multiple forms of the native receptor for a given steroid and may explain the difference of the action of a given steroid in anabolic

and catabolic target tissues (cortisol induced anabolic effects in the liver and catabolic effects of the same hormone in the thymocytes). In addition, it would also explain the tissue specific differences in the affinity, i.e. the capacity of receptor to bind steroid (for instance the difference in the affinity of receptors for cortisol binding in the liver and uterus).

The steroid receptor activation mechanism seem to involve phosphorylation. There are some observations suggesting the "autocatalytic" regulation of receptor phosphorylation.

Regulatory role of the receptor

The nature of other subunits of the receptor vary from tissue to tissue. We propose that some of them may be common for all steroid receptors. They seem to be involved in the control of post-transcriptional and translational regulation causing of chemical modifications of preformed molcules (phosphorylation, acetylation) and cell membrane transport. These modifications occurring upon the activation of the native receptor underlie the extragenomic effects. One or several subunits seem to be involved in the phosphorylation/dephosphorylation regulatory mechanism(s). The "model" postulates that in anabolic target tissues one of the receptor subunits might be either regulatory or catalitic subunit of protein kinase.

The phosphorylations independent of cAMP, observed after steroid administration, i.e. upon the "activation" of the receptor might suggest that the "catalytic subunit" of protein kinase might be one of steroid receptor subunits. The alternative possibility is that the observed phosphorylations might be attributed to a protein-specific modifier. This protein might serve as a receptor subunit and might be, upon the receptor activation, involved in the modifying the activity of preformed protein kinases. On the contrary, in catabolic target cells a specific phosphatase instead of phosphoprotein kinase might serve as a subunit of the steroid receptor.

The activities of both plausible subunits of receptor may link the activity of steroid receptor(s) with the activity of adenil-cyclase system. Released, upon the activation of the receptor the subunits seem to be involved in the basic phosphorylation/dephosphorylation regulatory mechanism. Even the translocation of "steroidophilic" subunits to the nuclei seems to be dependent on the phosphorylation controlled by the receptor activation.

Finally, it should be emphasized that the total integrated response of target cells to the respective steroid hormones is not only due, as the present "dogma" postulates, to the increased rate of transcription of a few particular structural genes, but that it also involves an immediate enhancement of the translation of preformed particular mRNAs, resulting in the synthesis (steroid-induced synthesis) of some early specific proteins, which seem to be regulatory proteins. This effect seems to be a short termed extragenomic effect, which is likely to be involved in the late gene expression, i.e. in the synthesis of some particular proteins on *de novo* synthesized mRNAs, i.e. in the expression of particular genes. Although the proposed "model" is highly speculative one requiring further experimental substantiation, it nevertheless provides a tentative link between extragenomic and genomic effects caused by steroids in the appropriate responsive tissues. Further theoretical elaboration and experimental substantiation of the basic concepts are now in progress in our laboratories.

Acknowledgements—This research project has in part been supported by the grant obtained from the Foundation of the Socialistic Republic of Serbia and the Grant from the Serbian Academy of Sciences and Arts. We are extremely grateful to Mrs Danka Filipović, Smiljana Naumović and Ankica Ledeci for their help in the preparation of this manuscript.

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Note added in proof

Our recent studies have revealed 3-fold increase of labelling of S-6 10 min after cortisol administration. The ^{32}P-orthophosphate was bound to serine. The amount of phosphoserine was significantly greater in 40 S basic proteins of cortisol treated animals (Kanasir *et al.* Submitted to *FEBS Lett.*, 1979).