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Activation of the Glucocorticoid–Receptor Complex

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A crucial step in the interaction of glucocorticoids with target cells is the activation step, which involves a conformational change in the cytoplasmic glucocorticoid–receptor protein complexes and facilitates their binding to the cell nucleus. Activation can be quantified by measuring the ability of glucocorticoid–receptor complexes to bind to polyanions, such as DNA-cellulose, and unactivated complexes can be separated from activated complexes by rapid ion exchange chromatography using diethylaminoethyl (DEAE)-Sephadex or DEAE-cellulose. Activation occurs in vivo under physiological conditions and the rate of activation of cytoplasmic glucocorticoid–receptor complexes can be enhanced in vitro by physical manipulations (elevated temperature, increased ionic strength, dilution). In vitro studies suggest that activation is a regulated process and a low molecular weight component termed *modulator*, which has been identified in rat hepatic cytosol, inhibits activation. Additional studies employing phosphatase inhibitors, such as molybdate, and purified calf intestinal alkaline phosphatase suggest that either the receptor protein or a regulatory component is dephosphorylated during activation. Results obtained with specific chemical probes suggest that activation results in the exposure of basic amino acid residues consisting minimally of lysine, arginine, and histidine. Pyridoxal 5′-phosphate, a specific probe for lysine residues, exerts dual effects on glucocorticoid–receptor complexes, since it stimulates the rate of activation and also inhibits the binding of previously activated complexes to nuclei or DNA-cellulose. The ability of 1,10-phenanthroline, a metal chelator, to inhibit the DNA-cellulose binding of activated complexes suggests that a metal ion(s) located at or near the DNA binding site may become exposed as a consequence of activation. Collectively, the results of these various experiments suggest that activation is a regulated biochemical phenomenon with physiological significance.

Key words: activation, glucocorticoid–receptor complex

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It is generally accepted that glucocorticoids, like other steroid hormones, exert their biological effects by combining with specific receptor proteins located in the cytoplasm of target tissues. These glucocorticoid–receptor complexes must then undergo a two-step process in order to bind to nuclei and ultimately regulate gene expression. The first step, termed "activation" or "transformation," involves a conformational change that results in an increased affinity of the hormone–receptor complex for nuclei and DNA. The second step, termed "translocation," involves the movement of the "activated" complex to the nucleus and its subsequent binding to acceptor sites. Since 1975 work in our laboratory has focused on probing the biochemical mechanism(s) underlying activation and the nature of the DNA-binding site that is exposed as a consequence of activation. From these biochemical studies a model of the activation step as a regulated process with physiological significance is beginning to emerge. It is not our intent here to review all of the published reports concerning various aspects of glucocorticoid–receptor activation extensively but rather to discuss the experimental results generated in our own laboratory with the appropriate supportive references.

OPERATIONAL DEFINITION OF ACTIVATION

Historically, the concept of glucocorticoid–receptor "activation" stemmed from studies dealing with the translocation and binding of these complexes to nuclei. Early studies performed in several laboratories demonstrated that when nuclei isolated from a variety of target tissues and cultured cells were incubated with radiolabeled glucocorticoid, very low levels of nuclear binding were observed. However, if the radiolabeled steroid was incubated first with cytosol at 0°C and then was incubated with nuclei at 20°C, a time-dependent specific nuclear binding of the radiolabeled glucocorticoid was detected [1–3]. It was clear from those early studies that the temperature-dependent reaction involves only a change in the cytosolic glucocorticoid–receptor complexes, since the nuclear binding occurs even at 0°C, provided that the labeled cytosol is heated at 20°C or 37°C prior to the incubation with nuclei [3–5]. Several laboratories reported on the absolute binding of the glucocorticoid to the cytosolic receptor prior to detection of nuclear binding. Milgrom and his colleagues [3] concluded that this effect is not simply a result of the stabilization of the receptor by the hormone, but that the binding of the glucocorticoid to the receptor is required before the presumed conformational change associated with "activation" can occur. Taken collectively, these studies suggested that activation can be defined in the physiological sense as the ability of glucocorticoid–receptor complexes to bind to nuclear acceptor sites.*

Since activation appears to involve a conformational change in the glucocorticoid–receptor complex resulting in the exposure of positively charged regions on the

* In this review we have used the term "activation" to refer exclusively to the changes in the glucorticoid–receptor complex that are required for binding of the complex to nuclei or polyanions. In contrast, Pratt and his colleagues [28,29] use this term to refer to the stabilization or regeneration of the glucorticoid binding site itself, and use the term "transformation" to refer to the subsequent changes that facilitate nuclear binding.

surface of the molecule [3], it is not surprising that these complexes display an increased affinity for natural and synthetic polyanions including chromatin [6,7], nucleosomes [8], purified DNA [1,3,9], DNA-cellulose [10,11], phosphocellulose [12], CM-Sephadex [3], ATP-Sepharose [13], and sulfopropyl-Sephadex and glass beads [3]. Because of the time and care required to prepare clean isolated nuclei, many laboratories have utilized the relatively simple and convenient DNA-cellulose binding assay to quantitate cytosolic activated glucocorticoid–receptor complexes. A careful comparison of binding of activated complexes to DNA-cellulose and homologous nuclei shows that in cases where only comparative observations are needed and a systematic and constant underestimation of the concentration of activated complexes can be tolerated, the DNA-cellulose binding assay is satisfactory [11]. However, one caution concerning the quantitation of activation by the DNA-cellulose binding assay should be mentioned. It has been reported that a number of endogenous inhibitors, both macromolecular and low molecular weight in nature, can block either the activation step (structural or conformational change) itself or the subsequent function of the activated complexes (binding to nuclei or DNA-cellulose) (see [14] for a review). Thus the inability of glucocorticoid–receptor complexes to bind to DNA-cellulose may reflect the fact that the structural or conformational changes associated with activation have been blocked (hence complexes are unactivated) or that the function (DNA-cellulose binding) of the activated complexes has been blocked by association with an inhibitor. Our laboratory frequently couples DNA-cellulose binding assays with chromatographic resolution of unactivated and activated complexes on anion exchange resins to distinguish between these two alternatives.

Two early studies employing diethylaminoethyl (DEAE)-cellulose [15] and DEAE-Sephadex [2] demonstrated a single chromatographic form of rat liver glucocorticoid–receptor complexes. The conditions employed in these studies favor activation and hence the single peak detected presumably represented activated complexes. Two laboratories subsequently reported the successful resolution of unactivated and activated complexes using rapid ion exchange chromatography. Our laboratory reported [16] that the more acidic unactivated rat liver glucocorticoid–receptor complexes, which do not bind to carboxymethyl (CM)-Sephadex or DNA-cellulose, are eluted from minicolumns of DEAE-Sephadex in 20 mM potassium phosphate buffer containing 0.4 M KCl. The less acidic activated glucocorticoid–receptor complexes, which bind to both CM-Sephadex and DNA-cellulose, are eluted from DEAE-Sephadex at a lower salt concentration (0.2 M KCl). Likewise Sakaue and Thompson [7] resolved unactivated and activated glucocorticoid–receptor complexes by chromatography on DEAE-cellulose with potassium phosphate as the eluting salt. Using this anion exchange resin the peak of radioactivity corresponding to the unactivated complexes (peak II), which do not bind to either chromatin or DNA, is eluted by 0.2 M potassium phosphate. Exposure of labeled cytosols to elevated temperature (20°C) or high salt (0.2–0.5 M KCl), which are conditions known to induce activation, resulted in a shift of bound [^{3}H]triamcinolone acetonide to a position that is eluted with 0.06 M potassium phosphate (peak 1). As previously mentioned, a combination of DNA-cellulose binding for quantitation and either DEAE-Sephadex or DEAE-cellulose chromatography for visualization of unactivated and activated glucocorticoid–receptor complexes has been utilized for investigating various aspects of the complex process of activation.

PHYSIOLOGICAL SIGNIFICANCE OF ACTIVATION

Although most of the published reports have dealt with the process of activation as achieved in vitro, two studies have clearly demonstrated that this process occurs in vivo under physiological conditions. In the first study, Munck and Foley [17] incubated (37°C) isolated thymus cells with [^{3}H]dexamethasone and at various time intervals (0.25–30 min) the cells were collected, cytosols prepared, and the proportion of unactivated versus activated receptor complexes was determined using chromatography on DEAE-cellulose as described previously [7]. The experimental results demonstrated that unactivated complexes are the first to appear but with increasing lengths of incubation are rapidly replaced by activated complexes and are undetectable after 30 min. Hence it was clear that unactivated complexes have an important physiological role and serve as obligatory intermediates in the formation of activated complexes.

Concurrently [18] our laboratory studied in vivo activation in the intact animal. In this study [^{3}H]triamcinolone acetonide was injected intraperitoneally into adrenalectomized rats and liver and kidney cytosols were prepared at different time intervals. Unactivated and activated glucocorticoid–receptor complexes were resolved by rapid chromatography on DEAE-Sephadex as described previously. In both liver and kidney cytosols unactivated complexes accounted for 40–50% of the bound radioactivity after 5 min, which was the earliest time measurement. Subsequently, until 60 min, a gradual decline in unactivated complexes occurred while the proportion of activated complexes increased. After 60 min the total bound radioactivity declined significantly, presumably as a result of nuclear translocation of activated complexes. Addition to the homogenization buffer of nonradioactive triamcinolone acetonide at 100-fold the in vivo dose of labeled hormone did not affect these chromatographic patterns, indicating that the activated complexes were not formed during the in vitro manipulations of labeled cytosols. This report from our laboratory confirmed that activation is a physiologically significant and relevant process.

FACTORS THAT AFFECT ACTIVATION IN VITRO

In vitro activation of the glucocorticoid–receptor complex occurs slowly at 0°C. Early studies showed that the rate of activation is enhanced by dilution, by gel-filtration or by an increase in either ionic strength or temperature [1,3,19,20]. The initial studies on nuclear translocation of the hormone–receptor complex did not treat activation as a discrete step and it was assumed that the only requirement for activation was binding of the steroid ligand to the receptor protein. It is now clear that activation of the hormone–receptor complex involves more than binding of the steroid to the cytoplasmic receptor. Recent studies indicate that activation of the glucocorticoid–receptor complex is a regulated process and suggest that the receptor or a regulatory component must be dephosphorylated in order for the hormone–receptor complex to assume the activated conformation.

Early studies from this laboratory demonstrated the presence of a low molecular weight component in rat liver cytosol that inhibited the binding of the glucocorticoid–receptor complexes to DNA-cellulose [19,21]. Because the same component appeared to be required to maintain the unoccupied receptor in a conformational state that was capable of binding [^{3}H]steroid, it was named *modulator*. The initial study defining

modulator was based on experiments in which it was shown that with continued elution of a gel filtration column (Bio-Gel P-4) overloaded with rat liver cytosol containing [^{3}H]glucocorticoid–receptor complexes, a decrease in receptor binding to DNA-cellulose was observed. Recently we have reexamined these column overload experiments [22]. An excess of rat liver cytosol containing [^{3}H]triamcinolone acetonide–receptor complexes was applied to a Bio-Gel P-4 minicolumn and eluted in 1-ml fractions. Selected fractions were assayed for their ability to bind to DNA-cellulose and, in addition, were chromatographed on DEAE-cellulose in order to ascertain the state of activation of the steroid–receptor complexes. Those fractions which exhibited the highest degree of DNA-cellulose binding also showed the largest peak corresponding to activated complexes on DEAE-cellulose (peak I eluted with 0.06 M potassium phosphate). Conversely those fractions that presumably coelute with low molecular weight components and exhibited decreased DNA-cellulose binding capacity showed a larger unactivated peak (peak II eluted with 0.2 M potassium phosphate). Partial characterization of modulator indicates that it is a negatively charged, heat stable (100°C, 30 min) molecule that is not removed by methylene chloride (and therefore is not a steroid) and that it has a molecular weight of approximately 1500. Thus modulator has many of the properties of the activation inhibitor described previously by Bailly et al [23] and of the heat stable factor recently characterized by Leach et al [24].

Our studies [25] on the effect of calf intestinal alkaline phosphatase on the rate of activation of the glucocorticoid–receptor complex were prompted by studies from Pratt's laboratory [26–29] and by studies by Munck et al [4] that suggested that phosphorylation of the receptor or of some regulatory component(s) was necessary in order for steroid binding to take place. We reasoned that if phosphorylation of the receptor (or a regulatory component, ie, *modulator*) is required for steroid binding, then activation of the hormone–receptor complex may involve a dephosphorylation reaction. Two lines of evidence are consistent with the hypothesis that a dephosphorylation reaction is involved in the activation mechanism. First, we showed that the rate of activation of [^{3}H]triamcinolone acetonide–receptor complexes was stimulated threefold by incubation with a highly purified preparation of calf intestinal alkaline phosphatase. Second, we showed that heat-induced activation of glucocorticoid–receptor complexes is blocked by addition of either sodium molybdate or sodium tungstate, both of which are potent phosphatase inhibitors [30].

The effect of exogenous alkaline phosphatase on activation of the hormone–receptor complex was shown both as an increase in DNA-cellulose binding [25] and as an increased conversion of unactivated to activated complexes as detected by chromatography on DEAE-cellulose (Fig. 1) [25]. The effect was clearly due to the phosphatase activity of the enzyme preparation and was not due to a minor contaminant. In summary our study [25] showed that heat denaturation of the alkaline phosphatase activity (90°C, 15 min) destroyed the activating capacity of the enzyme. More importantly stimulation of activation by the exogenous enzyme was prevented by 1 mM arsenate, a potent inhibitor of alkaline phosphatase enzymes. Neither 10 mM arsenate nor 10 mM levamisole, both of which are specific inhibitors of alkaline phosphatase enzymes [31], inhibited endogenous activation of the complex. Therefore we have concluded that the endogenous enzyme which activates the glucocorticoid–receptor complex is not alkaline phosphatase and we proposed that the effect of the exogenous enzyme was due to an associated protein phosphatase activity. Moreover,

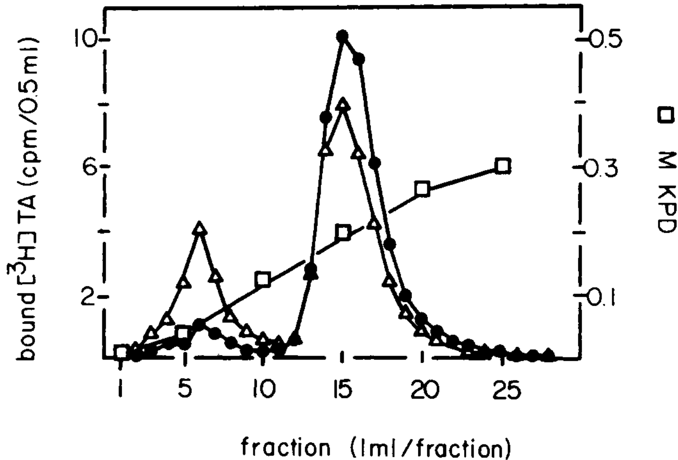

Fig. 1. Effect of calf intestinal alkaline phosphatase on formation of activated [^{3}H]triamcinolone acetonide–receptor complexes. Liver cytosol prepared in TSM buffer (0.05 M Tris-HCl, 0.25 M sucrose, 3 mM $MgCl_2$, pH 8.1 at 0–4°C) was incubated with 30 nM [^{3}H]triamcinolone acetonide at 0–4°C. At the end of the 2-hr incubation, desalted calf intestinal alkaline phosphatase was added to an aliquot of labeled cytosol at a final concentration of 452 μg/ml, and the incubation was continued for an additional 2 hr at 0–4°C. The cytosol was adjusted to 10 mM Na_2MoO_4 to prevent additional activation during chromatography and treated with dextran-coated charcoal; 0.3 ml was applied to a DEAE-cellulose column (3-ml bed volume). The columns were washed with 10 ml of equilibration buffer, pH 7.6, and the wash was discarded. The bound complexes were eluted with a 5–400 mM linear potassium phosphate gradient. Thirty 1-ml fractions were collected. (●) No additions; (△) incubation with calf intestinal alkaline phosphatase; (□) M KPD. Figure reproduced from Barnett et al [25].

the fact that relatively high, nonphysiological concentrations of the calf intestinal alkaline phosphatase are required to stimulate activation suggests that the component of the receptor system (presumably a protein) that is being dephosphorylated is not a preferred substrate for the calf intestinal enzyme. Recently it has been shown that the protein phosphatase activities associated with alkaline phosphatase enzymes from calf intestine, beef heart, and E coli dephosphorylate phospho-Tyr-histones at five to ten times the rate they dephosphorylate phospho-Ser-histones [32]. Rabbit muscle protein phosphatase preferentially dephosphorylate phospho-Ser-histones. It may be relevant that Weigel et al [33] have reported that both the purified A and B subunits of the avian progesterone receptor can serve as a substrate for the purified catalytic subunit of the beef heart cAMP dependent protein kinase using physiological concentrations of the enzyme. The phosphorylated progesterone receptor can then be dephosphorylated by bovine alkaline phosphatase [34]. Studies [35] show that the cAMP dependent protein kinase preferentially phosphorylates specific serine and threonine residues.

A second observation consistent with the hypothesis that activation involves a dephosphorylation mechanism is the demonstration that endogenous activation of the glucocorticoid–receptor complex is blocked by the phosphatase inhibitors sodium molybdate and sodium tungstate [30]. Our own experiments [25] show that addition of molybdate to rat liver cytosol prior to heat treatment prevents binding to DNA-cellulose. Addition of molybdate after heat-induced activation has no effect on subsequent DNA-cellulose binding. These results were extended to show that both

molybdate and tungstate prevent heat-induced formation of the activated complex as judged by chromatography on DEAE-Sephadex or DEAE-cellulose. Addition of molybdate (or tungstate) to unactivated or previously activated complexes prior to chromatography had no effect on their subsequent elution profiles.

We also have demonstrated that a variety of low molecular weight phosphoesters stimulate the rate of activation of glucocorticoid–receptor complexes. Presently these compounds appear to fall into one of the following groups: (1) compounds like pyridoxal 5′-phosphate that stimulate activation due to formation of a Schiff base; (2) synthetic phosphoesters, such as *p*-nitrophenyl 5′-phosphate and α-napthyl phosphate, that serve as alkaline phosphatase substrates, and (3) ATP or compounds which at substrate level concentrations create an ATP regenerating system in unfractionated cytosol.

Pyridoxal 5′-phosphate, previously shown to be an inhibitor of DNA-cellulose binding [36], has recently been shown to stimulate the rate of in vitro activation of the glucocorticoid–receptor complex [37]. As demonstrated (discussed later) for the effect on DNA-cellulose binding, the effect of pyridoxal 5′-phosphate on activtion is due to its ability to form a Schiff base. Pyridoxamine phosphate, pyridoxine, and pyridoxamine have no effect on activation and the dephosphorylated B_6 analog, pyridoxal, stimulates activation only at a fivefold higher concentration. These characteristics distinguish the effects of pyridoxal 5′-phosphate from the second group of phosphoesters, represented by *p*-nitrophenyl phosphate (PNPP) and α-napthyl phosphate, which also stimulate the rate of activation of the glucocorticoid–receptor complex. The PNPP-like compounds do not affect binding of the activated complexes to DNA-cellulose, do not form a Schiff base and do not appear to require a phosphate group for activity. *p*-Nitrophenol, the dephosphorylated analog of PNPP, is more potent than PNPP in stimulating the rate of activation of the hormone-receptor complex. How the PNPP-like compounds exert their effect is not known.

We have evidence that the stimulation of the rate of activation by ATP, which was first noted by John and Moudgil [38], is due to the unhydrolyzed triphosphate (unpublished results). ATP can be replaced by the hydrolysis resistant analogs, 5′-adenosyl methylenediphosphate (AMP-PCP) or 5′-adenyl methylenephosphonate phosphate (AMPCPP), demonstrating that activation by ATP does not involve either a phosphorylation or an adenylation reaction. GTP and its hydrolysis-resistant analog, 5′-guanosyl methylenediphosphonate (GMP-PCP), are equally effective. Although very high concentrations of ATP (15 mM at 15°C) are required to produce a maximal effect, examination of nucleotide metabolism by high performance liquid chromatography reveals that metabolism of ATP in liver cytosol is extensive (greater than 90% within 1 min at 4°C) and that the effective concentration of ATP that stimulates activation of the glucocorticoid–receptor complex in our experiments is within the physiologically relevant range (~1 mM). Dose-response curves show that optimal stimulation of activation by α-glycerophosphate, 3-phosphoglycerate, and 2-phosphoglycerate occurs at substrate level concentrations (30 mM) and suggest that these compounds exert their effect by creating an ATP regenerating system. A summary of the effects of these compounds on the activation step is presented in Table I.

Molecular weight estimates ranging from 66,000 to 300,000 have been reported for the glucocorticoid receptor [2,39,40]. The best studies indicate that in the presence of molybdate the large molecular weight forms are detected whereas molecular weight estimates of the activated receptor prepared in the absence of molybdate indicate a

TABLE I. Summary of Compounds That Affect Activation of the Glucocorticoid–Receptor Complexes

Compounds	Possible mechanism
Inhibitors of activation	
MoO_4, WO_4	Known phosphatase inhibitors
Stimulators of activation	
Calf intestinal alkaline phosphatase[a]	Associated protein phosphatase activity
ATP, AMP-PCP, AMPCPP, GTP, GMP-PCP[a]	Allosteric interactions with receptor or with a regulatory component
α-glycerophosphate, 3-phospho-glycerate, 2-phosphoglycerate[a]	Creation of ATP regenerating system
α- or β-naphthylphosphate, *p*-nitrophenylphosphate *p*-nitrophenol[a]	Alkaline phosphatase substrates; mechanism unknown
Pyridoxal 5′-phosphate[b]	Schiff base formation with lysine

[a]Stimulation blocked by MoO_4 or WO_4.
[b]Effects of MoO_4 and WO_4 have not been tested.

Stokes radius of 60 Å (90,000 molecular weight). It is not clear whether the difference in molecular weight between molybdate stabilized, unactivated complexes and activated, hormone–receptor complexes is due to a limited proteolysis, which is part of the activation mechanism, or whether the high molecular weight forms seen in vitro in the presence of molybdate represent a tendency of the unactivated complex to aggregate. Two lines of evidence suggest that activation of the glucocorticoid receptor is not the result of limited proteolysis. First, Sakaue and Thompson (personal communication) have found that a number of diverse proteolytic inhibitors including leupeptin, elastinal, antipain, phenylmethylsulfonyl fluoride, phosphoramidon, pepstatin, chymostatin, and trypsin inhibitor fail to block heat-induced activation of the rat liver glucocorticoid–receptor complex as assayed by DEAE-cellulose chromatography. Second, studies on intact cells show that following activation and translocation the receptor can be recycled from the nucleus back to the cytoplasm [41,42]. If intact cells are exposed to steroid, there is a decrease in the steroid binding activity of the cytoplasm and a concomitant increase in specifically bound steroid associated with the nucleus. Removal of the steroid from the incubation medium results in a decrease in nuclear-associated radioactivity and an increase in the steroid binding activity in the cytoplasm. The increase in cytoplasmic binding activity following steroid removal is an energy dependent process [41] and occurs in the absence of new protein synthesis [41,42]. These studies show that unlike proteolysis, the change in the receptor protein that occurs as a consequence of activation is reversible in vivo. Along these lines, a very recent report by Raaka and Samuels [43] utilizing density labelling of the glucocorticoid receptor with heavy isotope-substituted amino acids in GH_1 cells indicates an equilibrium between the high molecular weight unactivated receptor complex and the 4S activated receptor complex. After nuclear transfer of the 4S form and subsequent recycling into the cytoplasm, the high molecular weight unactivated form is reconstituted in the cytoplasm in the absence of new protein synthesis. Finally, the observation from our laboratory [21] that following in vitro activation the receptor does not rebind steroid argues that activation involves more than a steroid-induced

conformational change in the receptor protein and is consistent with the hypothesis that activation of the glucocorticoid–receptor complex is due to covalent modification of either the receptor protein or of some regulatory component. Indirect evidence, presented above, suggests that a dephosphorylation reaction is one step in the activation of the hormone-receptor complex.

CHARACTERIZATION OF THE DNA-BINDING SITE

The precise nature of the DNA-binding site that becomes exposed as a consequence of activation is not understood thoroughly. To this end our laboratory has studied the effects of a number of specific chemical probes on the binding of [^{3}H]triamcinolone acetonide to rat hepatic glucocorticoid receptors and on the binding of thermally activated [^{3}H]triamcinolone acetonide–receptor complexes to DNA-cellulose. DiSorbo et al [44] reported that if the glucocorticoid binding site was first protected with saturating levels of steroid, then 1,2-cyclo-hexanedione, a probe specific for arginine residues, inhibited the binding of activated complexes to DNA-cellulose without affecting ligand binding. Likewise ethoxyformic anhydride and the photoactivated oxidant, rose bengal, two chemical probes that selectively modify histidine residues, also inhibited the binding of activated complexes to DNA-cellulose while having little effect on steroid binding to receptor. As will be discussed subsequently in more detail, pyridoxal 5′-phosphate, which forms a Schiff base with the ϵ-NH_2 group of lysine residues, also was shown to inhibit the binding of activated complexes to DNA-cellulose. Taken collectively these data suggest that activation of hepatic glucocorticoid–receptor complexes results in the exposure of basic amino acid residues consisting minimally of lysine, arginine, and histidine. The effects of these and other chemical probes on the DNA-cellulose binding of activated receptor complexes are summarized in Table II.

Since it had been postulated that activation of glucocorticoid–receptor complexes results in the exposure of the positive charges of basic amino acids such as lysine, our laboratory [45] tested the effects of pyridoxal 5′-phosphate on the binding of activated complexes to DNA-cellulose. Our experiments demonstrated that pyridoxal 5′-phosphate can inhibit this binding, presumably by forming a Schiff base with the ϵ-NH_2 group of a lysine residue that is one of the required residues functioning in

TABLE II. Chemical Probes of the DNA-Binding Site of the Activated Glucocorticoid–Receptor Complex

Compound tested (concentration)	Specificity	Inhibition of DNA-cellulose binding of activated receptor complexes
Pyridoxal 5′-phosphate (10 mM)	Lysine residues	+
Pyridoxamine-phosphate (10 mM)	Inactive	−
Pyridoxine (10 mM)	Inactive	−
1,2-Cyclohexanedione (100 mM)	Arginine residues	+
Ethoxyformic anhydride (34 mM)	Histidine residues	+
Rose bengal (80 μM)	Histidine residues	+
1,10-Phenanthroline (3 mM)	Metal chelator	+
1,7-Phenanthroline (3 mM)	Nonchelating isomer	−

the DNA-binding site of the activated complex. Cake et al [36] subsequently reported that the pyridoxal 5′-phosphate effect is concentration dependent and specific for the physiologically active form of the cofactor. Pretreatment with pyridoxal 5′-phosphate also was shown to inhibit the binding of heat-activated receptor complexes to isolated rat liver nuclei and phosphocellulose. The observed inhibition of DNA-cellulose binding was found to be competitive with respect to DNA, suggesting that the effect of the cofactor is directly on the DNA-binding site of the activated glucocorticoid–receptor complex. Experiments in which a reducing agent, sodium borohydride ($NaBH_4$), was employed confirmed that pyridoxal 5′-phosphate binds to a site(s) that is inaccesible in the unactivated receptor complex but becomes exposed as a consequence of activation.

In addition to its use as a probe for the DNA-binding site, pyridoxal 5′-phosphate has been employed to elute activated complexes from DNA-cellulose and nuclei [46,47]. Cidlowski and Thanassi [46] reported that the release of nuclear dexamethasone–receptor complexes apparently depends on the integrity of the C4-carboxyaldehyde group of pyridoxal 5′-phosphate, since the extraction can be inhibited by either hydroxylamine or semicarbazide. Our laboratory [47] reported that the pyridoxal 5′-phosphate-eluted receptor complexes are less prone to aggregation at low ionic strength and more stable with respect to steroid binding than complexes eluted with 0.45 M NaCl. These results were consistent with an earlier report by Cidlowski and Thanassi [48] concerning the apparent conversion of unactivated complexes (7–8S) to a form that has a sedimentation profile similar to activated complexes (4–5S) after incubation with pyridoxal 5′-phosphate. Although these authors concluded that pyridoxal 5′-phosphate either induces changes in receptor conformation or disaggregation, our laboratory [37] has demonstrated recently that under similar experimental conditions pyridoxal 5′-phosphate significantly and specifically enhances activation as ascertained by chromatography on DEAE-cellulose chromatography. Thus it appears that pyridoxal 5′-phosphate exerts dual effects since it stimulates the rate of activation and inhibits the subsequent binding of activated complexes to DNA-cellulose. Since both of these effects are apparently dependent on Schiff base formation, lysine residues are implicated in the conformational change associated with activation as well as being involved at the DNA-binding site. Although the precise mechanism by which pyridoxal 5′-phosphate stimulates activation is unclear, this phenomenon may be explained most simply in terms of mass action. By binding to the activated glucocorticoid–receptor complexes, pyridoxal 5′-phosphate may effectively remove these complexes from the reaction and hence stimulate the formation of more activated complexes.

Two published reports from our laboratory suggest that pyridoxal 5′-phosphate actually may regulate glucocorticoid-receptor activity in vivo. DiSorbo et al [49] assayed the DNA-cellulose binding ability of hepatic glucocorticoid–receptor complexes from control and B_6-deficient animals. Their findings indicated that a reduction in the intracellular level of pyridoxal 5′-phosphate facilitated an increase in the number of glucocorticoid–receptor complexes capable of binding to DNA-cellulose in vitro. Subsequently DiSorbo and Litwack [50] reported that incubation of rat hepatoma cells (FAZA) in pyridoxine-free medium resulted in a decreased intracellular level of pyridoxal 5′-phosphate and significant enhancement of the induction of

tyrosine aminotransferase, which is a well described phenotypic response of hepatoma cells to glucocorticoids. Likewise the addition of pyridoxine to the culture medium significantly reduced induction of tyrosine aminotransferase. Since enzyme induction is a direct consequence of the binding of activated glucocorticoid–receptor complexes to nuclear acceptor sites, these results suggest that pyridoxal 5′-phosphate may regulate the functionality of these complexes in vivo.

Recently our laboratory has reported that the metal chelator, 1,10-phenanthroline, interacts with thermally activated (25°C for 30 min) glucocorticoid–receptor complexes and blocks their subsequent binding to DNA-cellulose [51]. In addition 1,10-phenanthroline causes the previously activated complexes to be eluted from DEAE-cellulose at a salt concentration similar to that at which unactivated complexes are eluted (0.2 M potassium phosphate). Thus operationally 1,10-phenanthroline appears to reverse activation. These results suggest that the glucocorticoid–receptor may be a metalloprotein and that a metal ion(s), which is located at or near the DNA-binding site, may become exposed as a consequence of activation. The observed inhibition of DNA-cellulose binding may be the result of either removal of a metal ion(s) from the receptor or formation of a complex between 1,10-phenanthroline and the receptor bound metal. Currently we are testing the latter hypothesis by investigating the interaction between highly purified activated glucocorticoid–receptor complexes and radiolabeled 1,10-phenanthroline.

SUMMARY AND CONCLUSIONS

Despite significant advances in the fields of biochemical endocrinology and cell biology the precise mechansism(s) by which glucocorticoid–receptor complexes regulate the expression of specific target cell genes is not totally understood. What has become clear is that a crucial aspect of this interaction of glucocorticoids with target cells is the activation step, which is required for nuclear binding of the steroid–receptor complex and occurs both in vitro and in vivo under physiological conditions. In this report we have attempted to summarize the various probes that our laboratory has utilized to study the biochemistry of activation. Based on our experimental data as well as that of other laboratories we have proposed a speculative model which is summarized in Figure 2. Published reports from the laboratories of Munck [4] and Pratt [26–29] suggest that phosphorylation of the receptor protein or of some regulatory component(s) is required in order for steroid binding to occur. Our laboratory has speculated that the subsequent activation step may involve a dephosphorylation reaction. The observed inhibition of activation by phosphatase inhibitors, such as molybdate, and the stimulation of activation by exogenous alkaline phosphatase support this hypothesis. Additional evidence also suggests that during activation a low molecular weight inhibitor termed modulator dissociates from the glucocorticoid–receptor complex. Results obtained with specific reagents such as pyridoxal 5′-phosphate and 1,10-phenanthroline have led us to speculate that the DNA-binding site, which is exposed as a consequence of activation, contains basic amino acid residues and possibly a metal ion(s). Obviously this is a relatively simplified model and future research in this field may reveal that activation is a very complex biochemical process that is controlled by multiple regulatory factors.

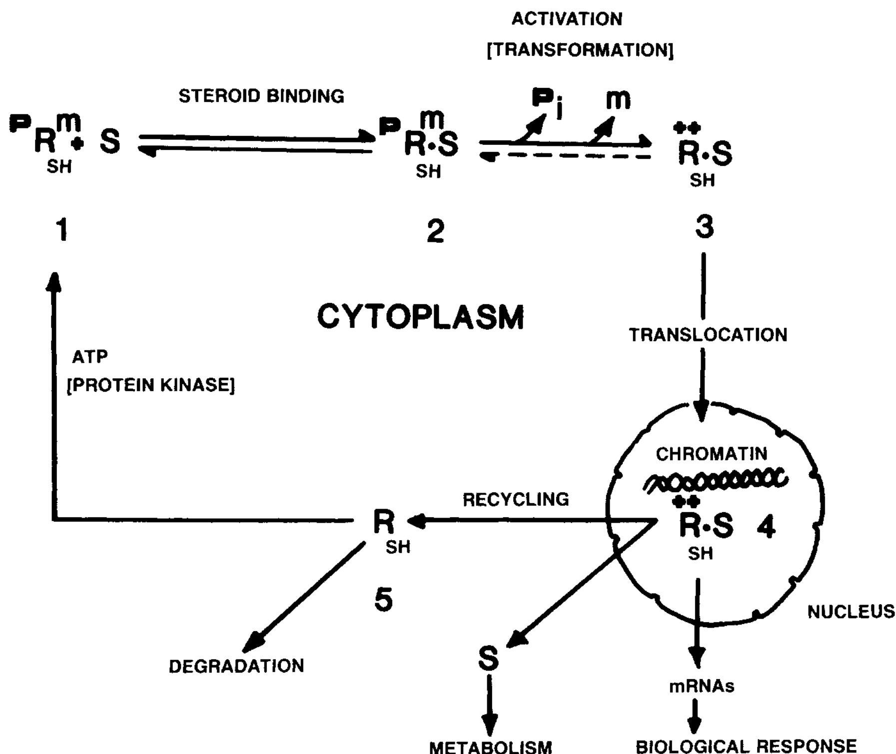

Fig. 2. Activation of the glucocorticoid-receptor complex—a speculative model. (1) Receptor (R), which is both phosphorylated (P) and contains reduced sulphydryl groups (SH), is capable of binding glucocorticoid (S). Unbound receptor may have associated with it a low molecular weight, heat-stable *modulator* (m) that maintains the protein in a conformation favorable for steroid binding and inhibits the subsequent activation. (2) Unactivated glucocorticoid-receptor complex. (3) Activated (transformed) glucocorticoid-receptor complex in which positively charged amino acid residues have been exposed at the DNA-binding site. (4) Activated glucocorticoid-receptor complexes that have translocated and associated with chromatin acceptor sites; and (5) Receptor protein that has been recycled from the nucleus back to the cytoplasm. It is not clear whether loss of steroid results in a conformational change that buries the positively charged residues. These residues, however, are clearly not exposed in the regenerated unbound receptor (1) which is again capable of rebinding steroid.

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