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THE HORMONE BINDING DOMAIN OF THE MINERALOCORTICOID RECEPTOR CAN REGULATE HETEROLOGOUS ACTIVITIES *in cis*

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SUMMARY: Steroid receptors are maintained inactive in the absence of cognate ligand partly because of repression by their hormone binding domain (HBD). Proteins complexed with the unliganded HBD of vertebrate steroid receptors, including the heat-shock protein 90, have been implicated as components of a molecular switch. As such, the HBDs of both the glucocorticoid and estrogen receptors have been shown to be autonomous regulatory cassettes which can subject heterologous activities resident on the same polypeptide to hormonal control. We show that the HBD of the mineralocorticoid receptor (MR) carries a similar "protein inactivation" function. Thus, the MR HBD can be used as a movable regulatory domain, a powerful tool for aldosterone regulation of chimeric proteins.

Steroid receptors are ligand-regulated transcription factors (reviewed in refs. 1-4). Their hormone binding domain (HBD) plays a key role as a molecular switch. The HBD seems to keep aporeceptors inactive as transcription factors by repressing one or several of the following essential functions (5, 6; and references therein): Dimerization, nuclear localization, DNA binding, and transcriptional regulation. Repression by the HBD of functions mapping outside of the HBD is relieved upon hormone binding or by deletion (see for example refs. 7, 8) of the HBD. In addition, a hormonally induced conformational change of the HBD may also unmask and / or induce certain functions which reside within the HBD itself. Notably, hormone-dependent dimerization (9-12), nuclear localization (11, 13) and transactivation functions (14-16) have been mapped to the HBD.

It has previously been shown that the "protein inactivation function" (5, 6, 17) associated with the HBDs of both the glucocorticoid receptor (GR) and the estrogen receptor (ER) could even subject a variety of heterologous proteins to hormonal control. As with wild-type receptors, the activity of a heterologous protein fused to a HBD is turned off in the absence of hormone and is induced very specifically and rapidly by hormone addition (for review, see ref. 18).

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Biochemical studies have established that all vertebrate steroid receptors are complexed with additional proteins including the heat-shock protein 90 (HSP90) in the absence of hormone (reviewed in refs. 19-21). Since these proteins appear to bind within the HBD, they have been proposed to mediate the "protein inactivation function" of the HBD (5, 6, 18). In this view, the hormonally-induced disruption of the complex with HSP90 results in the concomitant relief of repression.

Since many commonly used cell lines contain endogenous GR and ER, the availability of additional regulatory domains with different ligand specificities would provide an extremely valuable tool. We have therefore tested the HBD of the mineralocorticoid receptor (MR) for its ability to regulate heterologous activities.

MATERIALS AND METHODS

Plasmids: All GCN4 derivatives were expressed from expression vector VAO (13, 22).

To insert the coding sequences for the *Saccharomyces cerevisiae* protein GCN4 into the expression vector VAO, a *Bgl*II site was introduced just 5' of the initiator codon of GCN4 by PCR using the oligonucleotide 5'GGAGATCTATGTCCGAATATC3' as an upstream primer. Using this *Bgl*II site and the *Sac*I site just downstream of the stop codon of GCN4 in plasmid pSP64-Sc4380 (a gift from K. Struhl, Boston), GCN4 coding sequences were inserted at the *Bam*HI site of VAO to yield plasmid GCN4.

To construct plasmid GCN4.ER, the stop codon of GCN4 was replaced first with a *Sal*I site by PCR with the oligonucleotide 5'CCGTCGACCGTTCGCCAAC3' as a downstream primer and the GCN4 clone YCp88-GCN4 (23) as template. Plasmid GCN4.ER differs from plasmid GCN4 by the insertion of a *Sal*I-*Sac*I fragment carrying human ER sequences from FosER (24) using the newly generated *Sal*I site at the 3' end of the GCN4 coding sequence.

The construction of plasmid GCN4.MR required the fusion of the *Hinc*II (*Sal*I) site at the 3' end of the GCN4 sequence of GCN4.ER to the *Stu*I site (codon 685) of the rat MR sequences (25). Ligation into the *Bam*HI site of VAO was facilitated by insertion of a *Bgl*II site just downstream of the stop codon of MR by PCR using a degenerate oligonucleotide for the sequence 5'CAGATCTACTTTCGGTGAAAGTAAA GGGG3'.

The reporter plasmid (TRE)5TL contains the following elements in pUC8: (i) From plasmid p(TRE)5TKCAT (26) an AP1 binding site (TRE) pentamer as a *Hind*III-*Bam*HI fragment at -109 of the thymidine kinase (TK) promoter from Herpes simplex virus; (ii) The luciferase coding region and SV40 splice and polyadenylation sites from plasmid pSV232A L-AΔ5' (27). To allow ligation to the *Bgl*II site at +52 of TK, a *Bam*HI site was first introduced upstream of the luciferase ATG by subcloning a *Rsa*I-*Ssp*I fragment encompassing the entire luciferase coding body into the *Sma*I site of pUC18 (plasmid pluci).

Cell culture and transfection: Monkey COS-7 cells (28) were grown in Dulbecco's modified Eagle's medium lacking phenol red and supplemented with 5% fetal calf serum. The serum was treated with 20 mg/ml acid-washed charcoal (Sigma) for 90 min at 4°C and refiltered. Cells were transfected by the calcium phosphate coprecipitation technique as previously described (13). For a 60 mm dish, 1 μg of (TRE)5TL was mixed with 1 μg of GCN4 derivative and 3 μg of pUC18 as carrier DNA. Hormones were added from 1000-fold concentrated stock solutions in ethanol 24 hrs before harvesting the cells. Note that COS-7 cells, derived from CV-1 cells, do not express endogenous ER or MR.

Luciferase assays: Cell extracts for luciferase assays (27) were prepared 42 hrs after adding the DNA for transfection by freeze-thaw lysis and analysed as described (29). Within a given experiment, the same amount of protein was used for all samples.

RESULTS

Experimental system: The transcription factor GCN4 from *Saccharomyces cerevisiae* was chosen as a test protein. GCN4 is functional in yeast as well as in mammalian tissue culture cells (30). It presents several technical advantages over other previously used proteins: (i) GCN4 is a strong transactivator whose activity can easily be assayed in mammalian cells by cotransfection with an appropriate reporter gene; (ii) GCN4 binds DNA as a homodimer in the absence of any accessory protein (31); (iii) One of the important domains of GCN4, the leucine zipper, is within a few amino acids of the C-terminus and thus can be positioned very close to the regulatory HBD in a fusion protein. This may help to achieve tight regulation.

HBDs were fused to the extreme C-terminal end of GCN4. GCN4.MR and GCN4.ER denote the hybrid proteins consisting of full-length GCN4 fused to the HBD of the MR and ER, respectively. GCN4.ER was constructed as a positive control since the ER HBD was known to be able to subject heterologous proteins to hormonal control.

To analyse GCN4 activity, expression vectors for GCN4 derivatives were cotransfected with the reporter plasmid $(TRE)_5L$ into the monkey cell line COS-7. Reporter plasmid $(TRE)_5L$ contains five copies of an AP1 binding site upstream of the thymidine kinase promoter driving luciferase expression. Note that GCN4 binds and activates (30; see also below) at a TRE (TPA response element) which can be activated by the mammalian transcription factor complex AP1. Plasmid constructs are schematically represented in Fig. 1.

The MR HBD subjects GCN4 to hormonal control: All GCN4 derivatives were tested in parallel in a transient expression assay. To avoid induction by contaminating steroids in the tissue culture medium, cells were kept in medium without phenol red and supplemented with charcoal-treated serum. For induction of GCN4.ER and GCN4.MR, 0.1 μM ß-estradiol and 0.1 μM aldosterone, respectively, were added to the tissue culture medium. Reporter gene activation was determined by luciferase assays 42 hours after transfection.

The results of representative experiments are shown in Table 1. Wild-type GCN4 induced the reporter gene 22-fold on average. Both GCN4.ER and GCN4.MR showed activity exclusively in the presence of their cognate ligands. Indeed, both GCN4.ER and GCN4.MR remained completely inactive without hormone and were hormonally induced 15-fold and 11-fold, respectively. In the presence of hormone, GCN4.MR reached almost 60% of wild-type GCN4 activity.

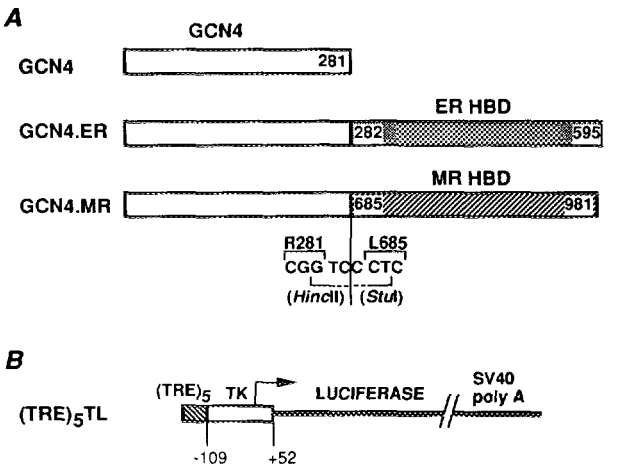

Figure 1. Diagrams of DNA constructs. (A) Schematic representations of the three GCN4 derivatives in expression vector VAO. The hormone binding domains of the estrogen (ER HBD; shaded box) and mineralocorticoid (MR HBD; hatched box) receptors are fused to full-length GCN4 (open box). Numbers within boxes indicate relevant amino acid positions of protein moieties. The details of the junctional sequences of GCN4.MR are shown. R281 is the C-terminal amino acid of GCN4 and L685 is the first amino acid of the MR HBD. (B) Reporter plasmid (TRE)5TL contains an AP1 binding site (TRE) pentamer (hatched box) linked to the thymidine kinase (TK) promoter (open box) at position -109, driving luciferase expression; the arrow indicates the transcription start site and direction.

An increased stability of the liganded chimeric proteins, which could partially explain the observed ligand-dependence, seems very unlikely for several reasons: (i) Relevant differences in stability have not been reported for other chimeric proteins

Table 1: Hormone-regulated GCN4 activity

Effector	Hormone	Expt. 1	Expt. 2	fold-induction
VAO	0	6	3	1x
GCN4	0	100	100	22x
GCN4.ER	0	7		
	100 nM ß	108		
GCN4.MR	0	5	5	1x
	1 nM aldo		48	
	100 nM aldo	60	52	11x
	0.1-1 µM spiro	8	2	
	1 nM aldo + 1 µM spiro		0	

Expression vectors for effector molecules were cotransfected with reporter plasmid (TRE)5TL into COS-7 cells. Luciferase assays were performed 42 and 24 hrs after adding DNA and hormone, respectively. Luciferase activities are expressed as % of GCN4 activity. The fold-inductions for GCN4 and GCN4.MR are relative to VAO and to no hormone, respectively. VAO, expression vector without GCN4 sequences; ß, ß-estradiol; aldo, aldosterone; spiro, spironolactone; Expt., experiment.

with the ER or GR HBDs; (ii) The addition of hormone had no effect on the levels of other hybrid proteins containing the MR HBD, notably a glucocorticoid-mineralocorticoid receptor chimera (32) and a ß-galactosidase-MR HBD fusion protein (data not shown).

Dose-response to agonist and antagonist: We determined the dose-response of the GCN4.MR protein by measuring luciferase activity from the reporter plasmid as a function of aldosterone concentration. The data shown in Fig. 2 indicate that the half-maximal response is reached at about 0.3 nM and that 0.5 nM aldosterone is sufficient to saturate the response. These values are compatible with the published aldosterone binding constant (K_d) of ~ 0.5 nM for the authentic rat MR (25). Although we have not directly determined the K_d of the GCN4.MR protein, the isolated MR HBD appears to maintain normal hormone binding affinity. Finally, we tested the ability of a mineralocorticoid antagonist to block the activation of GCN4.MR by aldosterone. While the antagonist spironolactone had no agonist effect by itself even at 1 µM, this concentration was sufficient to prevent the activation by 1 nM aldosterone. Thus, spironolactone also acts as an antagonist of aldosterone-mediated relief of repression by the HBD.

DISCUSSION

We have shown here that the HBD of the MR carries a "protein inactivation function" which is able to subject a heterologous protein to hormonal control *in cis*. This "inactivation function" is *on* in the absence of hormone and represses other activities

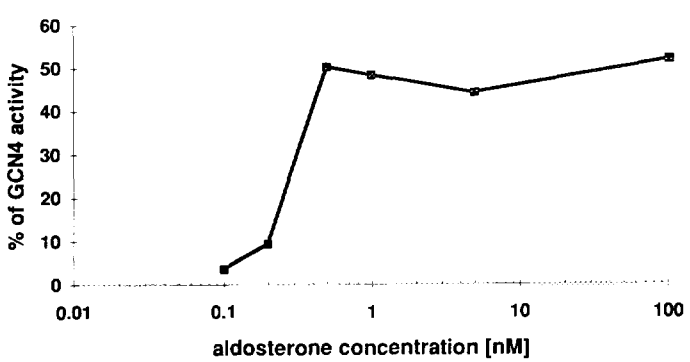

Figure 2. Dose-response of GCN4.MR. Luciferase activities obtained with GCN4.MR as a function of increasing aldosterone concentrations are indicated as % of the luciferase activity obtained with wild-type GCN4. Note that the X-axis has a logarithmic scale and that 0.1 nM gave the same response as no hormone.

resident on the same polypeptide. In the presence of an agonist, the "inactivation function" becomes turned *off* resulting in a relief of repression. We would like to emphasize that these findings, well beyond their technical importance, also suggest that the inactivation function of the HBD constitutes part of the molecular switch regulating aldosterone signalling by the wild-type MR.

The ability to inactivate heterologous proteins in a hormone-reversible fashion has thus been shown to be common to the HBDs of the GR (5), ER (17), and MR (this paper) receptors. The HBD of the MR will undoubtedly prove to be a very powerful tool. By analogy to the HBDs of the ER and the GR, the MR HBD is likely to be useful for the regulation of a wide variety of heterologous proteins. This is indeed confirmed by recent experiments in which we have been able to use the MR HBD for regulation of the serine / threonine kinase STE11 in yeast (J.-F. Louvion and D. Picard, unpublished results). It is of particular interest for applications in mammalian systems that the MR HBD can be induced by low physiological concentrations of aldosterone. Under these conditions, the endogenous GR, expressed in most tissues and cell lines, should not be induced (33).

Heterologous proteins can now be subjected to hormonal control by fusion to the HBD of either the GR, the ER, or the MR. With a choice of three regulatory domains, activation of endogenous steroid receptors in a particular cell line or tissue can be avoided. Moreover, different HBDs could be used for independent regulation of more than one heterologous protein in the same cell.

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