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LIGAND-INDUCED CONFORMATIONAL CHANGES IN THE MINERALOCORTICOID RECEPTOR ANALYZED BY PROTEASE MAPPING

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SUMMARY: The human mineralocorticoid receptor (MR) binds the agonists aldosterone and cortisol and the antagonist progesterone with a comparably high affinity. We used limited proteolysis of human MR synthesized by *in vitro* translation to detect structural alterations induced by these different endogenous ligands. Steroid binding induces a conformational change within the receptor protein. This structural alteration renders a fragment of MR resistent to proteolysis. Agonists and antagonist vary in how well they protect the MR fragment against proteolysis. But the two agonists also differ in their ability to protect, indicating that agonists and antagonists, but also different agonists, may induce distinct conformational changes. Ligand-independent removal of MR-associated heat-shock proteins induces no detectable structural change but completely prevents ligand binding of MR.

The mineralocorticoid receptor (MR) is a ligand-activated transcription factor (1, 2). In addition to the glucocorticoid receptor it mediates the genomic effects of corticosteroids (3). Molecular cDNA cloning and comparison of the amino acid sequence deduced revealed that MR belongs to the steroid hormone receptor superfamily, the members of which are all structured in a similar way and organized in different domains (1, 2, 4). In the absence of ligand, MR is thought to be complexed with heat-shock proteins (HSPs; HSPs 90, 70 and 56) and other less well characterized proteins that maintain the receptor in an inactive state. Binding of the steroid to this receptor complex is followed by the dissociation of MR from the inhibitory hetero-oligomeric structure (5, 6). After nuclear translocation, MR binds to steroid response elements in the regulatory region of target gene promotors either as a homodimer or together with the glucocorticoid receptor as a heterodimer and regulates gene expression (7). But only agonist-occupied MR has transcriptional activity; antagonist-

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Abbreviations: A, aldosterone; C, cortisol; HSP, heat-shock protein; MR, mineralocorticoid receptor; P, progesterone.

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occupied MR is not able to transactivate (8). One speculative explanation for this observation is that agonists and antagonists induce distinct structural alterations in MR.

Human MR binds both endogenous agonists aldosterone (A) and cortisol (C) with a comparably high affinity (1, 8, 9). As a consequence, in neuronal tissue MRs are nearly completely occupied by circulating C. In visceral tissues such as the kidney, however, MR is selectively activated by A. This is achieved by the cell-specific expression of 11ß-hydroxysteroid dehydrogenase, which is abundant in kidney but absent in neuronal tissue. This enzyme metabolizes C to cortisone, which has negligible affinity for MR. In this way MR is protected against C-overload, and its activation becomes dependent on the secretion of A (10, 11).

In the present study, we investigated structural alterations in human MR induced by the endogenous high-affinity ligands A, C and progesterone (P) by protease mapping. In addition, we examined the involvement of HSPs in conformational changes and ligand binding of MR.

MATERIALS AND METHODS

Materials: D-aldosterone, cortisol, progesterone and the proteases trypsin, chymotrypsin and subtilisin were obtained from Sigma (Deisenhofen, Germany). Radiolabeled L-[^{35}S]methionine (1000 Ci/mmol) was purchased from Amersham (Braunschweig, Germany) and RNAse inhibitor from Boehringer Mannheim (Mannheim, Germany).

Plasmids: Plasmid pGEM4-hMR containing the human MR coding sequence under the control of the T7 promotor was constructed by deleting an AccI-restriction fragment from the plasmid pGEM4-hk10, the construction of which has been reported previously (1).

Coupled *in vitro* transcription and translation: Circular plasmid (pGEM4-hMR) was transcribed and translated simultaneously in rabbit reticulocyte lysate in the presence of L-[^{35}S]methionine according to the instructions of the manufacturer (Promega, Madison, WI), except that the translation mixture contained 1µM zinc chloride.

Limited proteolytic digestion: Limited proteolysis experiments were performed using 5-µl aliquots of [^{35}S]methionine-labeled translation mixture. After a 10-min incubation period in the presence or absence of hormone, 1 µl of the protease was added and the incubation was continued for 20 min at 25°C. The digestion was stopped by removing and mixing 2 µl with 20 µl of denaturing loading dye. Samples were heated for 5 min at 95°C and loaded on 10% SDS polyacrylamide gels (12). Following electrophoresis, gels were dried under vacuum and autoradiographed. Some of the autoradiographs were scanned using a computer-assisted image analysis system (Optimas, Bioscan) to quantify the appearance of the ligand-protected proteolytic fragment.

RESULTS AND DISCUSSION

To investigate conformational changes in human MR we used limited proteolysis of *in vitro*-ranslated [^{35}S]methionine-labeled receptor protein. This method has been shown to be useful in detecting structural alterations within proteins (13-16). Nuclear receptors translated *in vitro* in rabbit reticulocyte lysate are well characterized and in terms of ligand- and DNA-binding properties they are similar to native receptors (14-17). Therefore, *in*

vitro-translated and [^{35}S]methionine-labeled MR is a useful tool for investigating interactions between ligand and receptor.

In vitro transcription and translation of pGEM4-hMR in the presence of [^{35}S]methionine resulted mainly in the production of a polypeptide of approximately 110 kDA, corresponding to the size of human MR (1) (Fig. 1). No labeled polypeptide was obtained when unprogrammed rabbit reticulocyte lysate was used (Fig. 1). After incubation with vehicle, A, C or P, labeled MR was digested with different amounts of protease and the products of the proteolysis were analyzed by denaturing electrophoresis. Digestion of MR without ligand was complete, whereas digestion of ligand-occupied receptor with chymotrypsin yielded a 35-kDa fragment and a 32-kDa fragment, with trypsin a 35-kDa fragment and with subtilisin a 32-kDa fragment when a low concentration of these proteases was used. However, the ability of the steroids to protect the fragment(s) against proteolysis differed considerably and became more distinct as increasing concentrations of protease were used. At high concentrations, protection of fragment(s) was best when MR

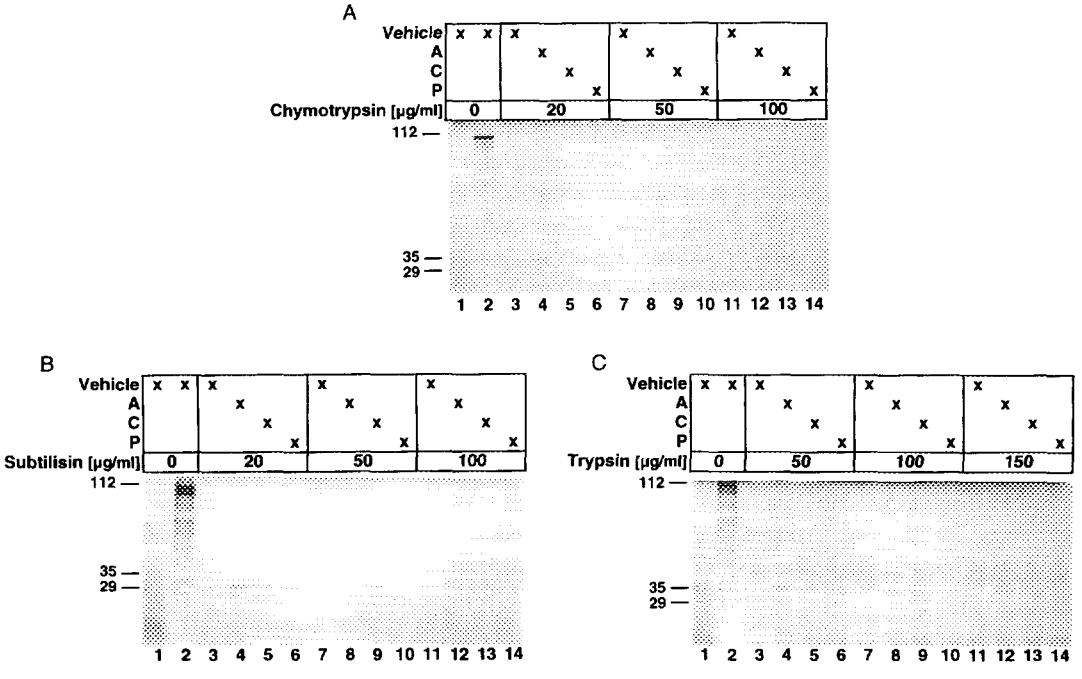

Figure 1. Agonist- and antagonist-induced conformational changes. MR translated *in vitro* was incubated with 100 nM A, C or P or with vehicle and digested with increasing concentrations of chymotrypsin (A), subtilisin (B) or trypsin (C). Lane 1 represents unprogrammed rabbit reticulocyte lysate, lane 2 undigested [^{35}S]methionine-labeled MR and lanes 3-14 proteolysis products of chymotrypsin (A), subtilisin (B) or trypsin digestion (C) of [^{35}S]methionine-labeled MR. The migration of molecular weight marker is indicated on the left.

occupied by A was the target of proteolysis. It was significantly less good when MR was occupied by C and was poorest when MR was occupied by the antagonist P (Fig. 1+2). Similar differences in the amount of fragments were obtained when lower concentrations of A, C and P were used (data not shown).

These data provide evidence that cognate ligands induce a structural alteration in human MR. Within the progesterone receptor and the glucocorticoid receptor (14, T. Trapp, unpublished observation) agonists and antagonists induce distinct conformational changes, since proteolysis of the antagonist-occupied receptor results in a smaller fragment than proteolysis of the agonist-occupied receptor. In the case of MR, there are no differences in the size of agonist- and antagonist-protected fragments. However, since the amount of protected fragment after digestion differs considerably between agonist- and antagonist-occupied MR it can be presumed that different structural alterations are induced by agonists and antagonists, leading to different availabilities of MR for digestion by proteases. Furthermore, also the agonists A and C differ in the degree to which they protect the MR fragment(s) against proteolysis. This indicates that there may be slight structural differences between A- and C-occupied MR. Therefore, A and C seem to influence MR in distinct ways even though they have the same affinity. These results raise the possibility that in addition to the metabolism of C by 11β-hydroxysteroid dehydrogenase, which protects MR against

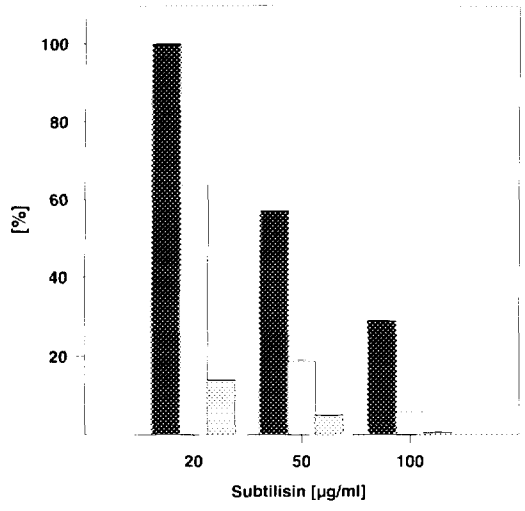

Figure 2. Quantification of proteolysis-protected fragment. The band representing the 35-kDa fragment protected against subtilisin-proteolysis (Fig. 1B) was densitometrically scanned and quantified. The optical density of the band obtained by digestion of A-occupied MR with 20 µg/ml subtilisin was set as 100%. Black, white and gray bars represent quantification of proteolysis products of A-, C- and P-occupied MR, respectively.

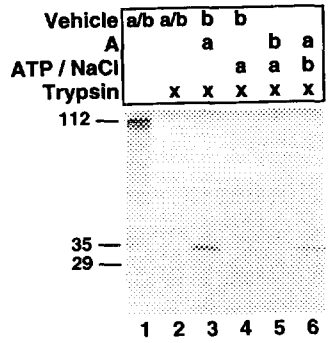

Figure 3. Conformational change is not induced by heat-shock protein release. Before proteolysis with trypsin (50 µg/ml) MR was incubated for two consecutive 10-min periods of time. For the first period (a) it was incubated with vehicle (lanes 1 and 2), 100 nM A (lanes 3 and 6) or 400 mM NaCl/10 mM ATP (lane 4 and 5) and for the second period (b) with vehicle (lanes 1, 2, 3 and 4), 100 nM A (lane 5) or 400 mM NaCl/10 mM ATP (lanes 6). Lane 1 represents vehicle-treated undigested [^{35}S]methionine-labeled MR and lanes 2-6 proteolyzed [^{35}S]methionine-labeled MR. The migration of molecular weight marker is indicated on the left.

C-overload, an intrinsic preference of MR for A over C may account for the A-selectivity in mineralocorticoid target tissue. This is consistent with recent findings showing that inhibition of 11β-hydroxysteroid dehydrogenase in a kidney cell system does not prevent the functional preference of MR for A over C (18).

As native steroid receptors, in rabbit reticulocyte lysate-translated steroid receptors are also associated with HSPs (19-22). To investigate the involvement of HSPs in conformational changes and ligand binding of MR we performed trypsin proteolysis after ligand-independent removal of HSPs by high concentrations of NaCl and ATP, a treatment known to segregate the HSP-receptor complex (22). Digestion yielded a 35-kDa proteolysis-resistant fragment after incubation with A (Fig. 3, lane 3), whereas MR digestion was complete without hormone (Fig. 3, lane 2). Neither dissociation of HSPs from the receptor with NaCl and ATP, nor incubation of MR with A after ligand-independent HSP removal protected the fragment against proteolysis (Fig. 3, lane 4 and 5, respectively), whereas incubation of MR with A before NaCl/ATP-treatment did (Fig. 3, lane 6). These data indicate that receptor-associated HSPs are necessary to enable MR to bind steroids with high affinity and that the structural alteration of the MR that occurs after steroid binding is not induced by HSP dissociation.

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