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By

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FKBP51, a new aldosterone induced gene in intestinal epithelium.
Mode of induction and possible roles.

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I would like to thank:

Reem- for his endless support.
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**Abbreviations**

11β-HSD2 11β-hydroxysteroid dehydrogenase type 2
ADX adrenalectomized
ANG II Angiotensin II
AngII-AVP Angiotensin II-Vasopressin dual receptor
receptor
AR androgen receptor
ASDN aldosterone-sensitive distal nephron
ATP adenosinetriphosphate
CBX carbenoxolone
CCD cortical collecting duct
CDC37 cell division cycle 37 homolog
CHIF corticosteroid hormone-induced factor
CHX cycloheximide
cDNA copy DNA
DMSO dimethylsulfoxide
DTT dithriothreitol
EGFP enhanced green fluorescent protein
ENaC epithelial sodium channel
FBS fetal bovine serum
FK506 Tacrolimus
FKBP51 51 kDa FK506-binding protein
FKBP52 52 kDa FK506-binding protein
GAPDH glyceraldehyde 3-phosphate dehydrogenase
GILZ glucocorticoid-induced leucine zipper
GR glucocorticoid receptor
GRE glucocorticoid responsive element
Hoxb7-EGFP transgenic mice that express EGFP under the direction of a homeobox B7 promoter/enhancer.
HRP horseradish peroxidase
Hsp90 heat shock protein 90
Hsp70 heat shock protein 70
Isc short-circuit current
IkBα inhibitor of NF-κB alpha
IKKα inhibitor of NF-κB kinase subunit alpha
M-1 mouse CCD derived cell line
mCCD(c1) mouse CCD derived cell line
mRNA messenger RNA
MR mineralocorticoid receptor
NADPH oxidase nicotinamide adenine dinucleotide phosphate-oxidase
Na+/K+-ATPase sodium potassium adenosinetriphosphatase
NF-κB nuclear factor kappa-light-chain-enhancer of activated B cells
Nedd4 neural precursor cell expressed, developmentally down-regulated 4
NCC Na+-Cl+ co-transporter
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>Kd</td>
<td>dissociation constant</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PEG300</td>
<td>polyethylene glycol 300</td>
</tr>
<tr>
<td>PLK2</td>
<td>polo-like kinase 2</td>
</tr>
<tr>
<td>PLZF</td>
<td>promyelocytic leucin zink finger</td>
</tr>
<tr>
<td>PPIase</td>
<td>peptidyl-prolyl cis–trans isomerase</td>
</tr>
<tr>
<td>PVDF</td>
<td>polyvinylidene fluoride</td>
</tr>
<tr>
<td>RT PCR</td>
<td>reverse transcription PCR</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodycyl sulfate</td>
</tr>
<tr>
<td>SGK</td>
<td>serum and glucocorticoid-induced kinase</td>
</tr>
<tr>
<td>shRNA</td>
<td>short hairpin RNA</td>
</tr>
<tr>
<td>SRE</td>
<td>steroid responsive element</td>
</tr>
<tr>
<td>TER</td>
<td>transpithelial resistance</td>
</tr>
<tr>
<td>TPR</td>
<td>tetratricopeptide repeat</td>
</tr>
<tr>
<td>Tris</td>
<td>tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>Usp2-45</td>
<td>ubiquitin-specific protease 2-45</td>
</tr>
<tr>
<td>XTT</td>
<td>2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide</td>
</tr>
<tr>
<td>Wnk1</td>
<td>WNK lysine deficient protein kinase 1</td>
</tr>
<tr>
<td>V_{max}</td>
<td>maximum rate</td>
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Summary

New aldosterone-regulated genes were identified by microarray analysis of distal colon epithelium isolated from adrenalectomized rats treated with aldosterone vs. vehicle. FKBP51, an Hsp90-binding immunophilin found in steroid receptor complexes and the IKKα complex, was found to be strongly induced by the mineralocorticoid treatment. Induction of FKBP51 in colon was confirmed by real-time PCR and Western blotting of colonic preparations. Ex-vivo measurements in colonic preparations have characterized the time course, dose-response and receptor specificity of FKBP51 induction. FKBP51 mRNA is up-regulated at physiological concentrations of aldosterone in a late (greater than 2.5 hours) response to the hormone. The induction of FKBP51 mRNA is at least partly inhibited by the protein synthesis inhibitor cycloheximide, suggesting an indirect action through other aldosterone regulated genes. Maximal increase in FKBP51 mRNA requires aldosterone concentrations that are higher than those needed to fully occupy the mineralocorticoid receptor (MR). Yet, induction of FKBP51 was fully inhibited by the MR antagonist spironolactone and insensitive to the glucocorticoid receptor (GR) antagonist RU486. In addition, at low concentration of aldosterone RU486 stimulated increase in FKBP51. The data suggest an essential role for MR in the induction of FKBP51 but requirement for GR occupancy as well. Several potential underlying mechanisms have been explored. The most likely one is that FKBP51 is activated by MR-GR hetero-dimer. Co-IP experiments using colonic protein extracts confirmed the existence of MR-GR dimers in this tissue.

To explore possible roles of FKBP51 in the response to aldosterone, a mineralocorticoid responsive cell line was constructed by stable transfection of the kidney collecting duct line M1 with MR construct. It was found that FKBP51 over-expression augments the expression level of MR while FKBP51 silencing reduces it in M1 cells. The data suggests a role of FKBP51 in maturation of MR complexes during aldosterone response. Measurements in M1-MR cells demonstrated that FKBP51 over-expression is without effect on the aldosterone-induced increase in Na⁺ transport. On the other hand, silencing endogenous FKBP51 has a mild inhibitory effect on the aldosterone response which could be attributed to the reduction in MR level.
Finally the possibility that the role of FKBP51 is to protect cells from oxidative cytotoxicity that will accompany the response to aldosterone, has been tested. Silencing FKBP51 in M1-MR cells did indeed increase their peroxide induced cytotoxicity. Thus, protecting cells from oxidative stress that is secondary to the aldosterone-induced increase in Na$^+$ transport and ATP consumption could be another role of this protein.
1. Introduction

1.1. Physiological role of Aldosterone.

1.1.1. Classical role of Aldosterone in epithelial tissues.
A mineralocorticoid Aldosterone is the final endocrine signal in the renin-angiotensin-aldosterone system that targets epithelia in the kidney and colon to regulate Na\(^+\) (re)absorption and K\(^+\) secretion (I). As a result, water follows net salt movement via osmosis, establishing maintenance of blood pressure. Angiotensin II (ANG II) and K\(^+\) promote aldosterone secretion (2,3). Decreased blood pressure increases ANG II production. Therefore, aldosterone secretion is increased as a result of decreases in blood pressure and increases in K\(^+\) and it is inhibited when blood pressure is increased and K\(^+\) is decreased.

1.1.2. Non-classical role of Aldosterone.
Aldosterone exerts its actions through the mineralocorticoid receptor (MR), a ligand-dependent transcription factor belonging to the nuclear receptor superfamily. MR is expressed in polarized epithelial tissues, such as the distal part of the nephron, the distal colon, and the salivary glands (4). These tissues are considered the classical aldosterone target tissues, where MR regulates ion and water homeostasis (5,6). However, MR is also expressed in non-epithelial tissues which are not primarily involved in sodium transport e.g. the heart, hippocampus, blood vessels, adipocytes and macrophages (7-10). Aldosterone was demonstrated to be implicated in the pathophysiology of cardiac fibrosis and cardiac hypertrophy in end-stage heart failure (11). The clinical relevance of MR in the pathogenesis of cardiac dysfunction was demonstrated by two large clinical trials in which patients with heart failure or post-acute myocardial infarction who received in addition to their usual treatment regimen low doses of MR antagonists showed a 30% reduction in morbidity and mortality (12,13). Accumulating data also indicate that aldosterone promotes tissue inflammation and fibrosis and plays a role in cell proliferation and apoptosis (14-17).
1.2. Mechanism of Aldosterone action.

1.2.1. Classical model of Aldosterone action.

The classical actions of aldosterone are mediated by intracellular receptors that translocate to the nucleus upon ligand binding. The activated MR and glucocorticoid receptor (GR), modulates gene expression by functioning as transcription factors via direct interaction with DNA binding sites [referred to as steroid response elements (SREs)]. In the absence of ligand the corticosteroid receptor is associated with a multi-protein complex of chaperones including Hsp90/ Hsp70 and immunophilins, which stabilize the receptor in an inactive but ligand-affine conformation. Upon ligand binding associated chaperones are released and receptor is translocated into the nucleus where it binds as a homodimer or heterodimer to inverted repeat DNA half sites in the promoter of target genes, activating or repressing their transcriptional activity.

Illustration 1 depicts aldosterone action in the classical aldosterone-sensitive tight epithelium, the principal cell of the renal collecting duct. Monolayers of these cells serve two primary and related functions: acting as barriers separating the internal and external environments and allowing (re)absorption of Na\(^+\)and water. Transcellular transport across these cells is electrogenic and dependent on serosal Na\(^+\)/K\(^+\)-ATPases that establish the electrochemical driving forces necessary for luminal entry and exit of Na\(^+\) and K\(^+\), respectively. The limiting step in Na\(^+\) (re)absorption is the activity of the luminal amiloride-sensitive epithelial Na\(^+\) channel (ENaC). ENaC activity, trafficking, and expression are important targets of regulation by aldosterone. Aldosterone can enhance transport by several mechanisms: 1) by promoting channel insertion and its proteolytic activation to increase the number of functional channels at the plasma membrane, 2) by increasing the open probability of apical ion channels, and 3) by increasing the level of ENaC subunits expression (18-20). Aldosterone also increases Na\(^+\)/K\(^+\)-ATPase activity to augment transport capacity (21, 22).

In recent years non-classical rapid effects of aldosterone, insensitive to transcriptional and translational inhibitors, were demonstrated. These non-genomic effects take place within minutes following aldosterone stimulation and involve second messenger signaling pathways. To date it is not clear whether these rapid actions are mediated by MR or by a putative novel transmembrane receptor (23).
Illustration 1: Model of the genomic actions of aldosterone (ALDO) in epithelial cells. Asterisks denote final effectors of aldosterone involved in Na$^+$ and K$^+$ reabsorption and secretion, respectively. The aldosterone-induced proteins serum and glucocorticoid-inducible kinase (Sgk), corticosteroid hormone-induced factor (CHIF), and Kirsten Ras (Ki-Ras) increase the activity and/or number of these transport proteins. ENaC, epithelial Na$^+$ channel; Hsp, heat shock protein; MR, mineralocorticoid receptor; GR, glucocorticoid receptor; SRE, steroid response element; 11β-HSD2, 11-β-hydroxysteroid dehydrogenase type 2 (Image taken from(24)).

1.2.2. Fast vs. slow effects of Aldosterone.

Early studies in our laboratory demonstrated that aldosterone elevates the apical Na$^+$ permeability of target epithelia by two different mechanisms: a relatively fast effect (less than 3 hr), and a slower or later (greater than 3 hr) response (25). The fast action is thought to be mostly mediated by the induction/repression of regulatory proteins that control the function of preexisting transporters such as ENaC, the Na$^+$/K$^+$-ATPase and K$^+$ channels. The latter response (>3 hours) is mediated by induction of components of the ion transport machinery itself (e.g. subunits of ENaC or the Na$^+$/K$^+$-ATPase) and genes that encode regulatory proteins that are likely to limit the aldosterone response (20). In agreement, it has been demonstrated that aldosterone binds to two different receptors in target epithelia: a high-affinity low capacity (K$_{1/2}$=0.5-3 nM), type I receptor (MR) and a low-affinity high capacity (K$_{1/2}$=90-150 nM) type II receptor (GR). At low doses aldosterone elicits a fast response of transient increase of sodium transport (peaking at 3
h) while at high doses aldosterone stimulates an approximately threefold larger, long lasting response (26). Within the physiologic range of aldosterone concentrations, sodium transport is predicted to be controlled by MR occupancy during circadian cycles and by MR plus GR occupancy during salt restriction or acute stress.

1.2.3. Selectivity of MR towards aldosterone in epithelial cells.

_In vitro_ studies have demonstrated that glucocorticoids can bind to and activate MR with the same affinity as mineralocorticoids. Since their plasma concentration normally exceeds that of aldosterone by a factor of 10–1,000 the outcome should have been permanent activation of MR by circulating glucocorticoids. This physiologically unfavorable scenario is prevented by the action of 11-β-hydroxysteroid dehydrogenase-2 (11βHSD-2) which is expressed at high abundance in aldosterone-responsive epithelia. 11βHSD-2 metabolizes hydroxy-glucocorticoids to inactive ketoglucocorticoids and thereby prevents their binding to MR (27). In addition, the inactive metabolites (11-dehydrocorticosterone in rodents and cortisone in human) can function as MR antagonists (28). The implication would be that a mineralocorticoid response will also depend on low level of circulating glucocorticoids.

1.2.4. Aldosterone induced genes modulating sodium retention.

**Early aldosterone-responsive genes.**

Several studies using candidate gene approaches or unbiased screens have been undertaken to discern the mechanistic basis of aldosterone action. As yet, only few early aldosterone-induced regulatory proteins have been identified and experimentally shown to have a positive effect on ENaC mediated Na⁺ transport. These are the serum and glucocorticoid-regulated kinase1 (Sgk1), G protein K-Ras2, the glucocorticoid-induced leucine zipper (GILZ), and the ubiquitin-specific protease Usp2-45 (29-32). In addition, the kidney-specific short form of lysine deficient protein kinase 1 (Wnk1) that stimulates Na⁺ reabsorption via the Na⁺-Cl⁻ co-transporter (NCC) and the corticosteroid hormone-induced factor (CHIF), which regulates the activity of the Na⁺/K⁺-ATPase were demonstrated to be regulated by aldosterone (33-35).

An important mechanism by which Sgk1 augments ENaC cell-surface expression is phosphorylation of the ubiquitin-protein ligase Nedd4-2. This phosphorylation interferes
with the ubiquitylation and subsequent degradation of ENaC mediated by this ligase (36, 37). The short signaling cascade that leads from Sgk1 induction via inhibition of Nedd4-2 to the activation of ENaC represents the first direct link between the aldosterone-regulated transcriptional activity of the mineralocorticoid receptor and the function of ENaC that has been demonstrated. However, while mice lacking MR are not viable, Sgk1 null mice show only mild abnormalities in sodium homeostasis, suggesting that other genomic targets are important for the overall regulation of sodium transport, as well as the existence of alternative pathways (38). This conclusion is further supported by the finding that transgenic mice carrying truncated ENaC that does not bind Nedd4-2 can respond to aldosterone (39). Recently Usp2-45 was also identified as an aldosterone induced regulator of ENaC that stimulates deubiquitylation of this channel.

In addition to the ubiquitylation route of regulating ENaC surface expression the RAS-RAF-MEK-ERK pathway was also shown to regulate ENaC. ERK appears to inhibit ENaC via its phosphorylation, which stimulates interaction with Nedd4 ubiquitin ligases (40, 41). Several proteins which specifically regulate the activity of that pathway have been reported to be aldosterone-regulated genes. Among these GILZ1 was shown to inhibit RAF and thus to stimulate Na⁺ transport (31, 42).

Recently, promyelocytic leukemia zinc finger protein (PLZF) was shown to be induced by short-term aldosterone stimulation and to inhibit ENaC mediated sodium transport by suppression of the mRNA levels of beta- and gamma-ENaC subunits (43). However, currently little is known about the negative feedback regulation of aldosterone signaling.

**Late aldosterone-responsive genes.**

The latter response to aldosterone is mediated by induction of components of the ion transport machinery itself. ENaC is composed of three distinct but similar subunits (α, β and γ) and located in the apical membrane of epithelial cells. Only a complex having all three subunits is a functional, Na⁺ conducting channel. The expression of all three ENaC subunits is stimulated in a tissue specific manner by a long term exposure to aldosterone. In the kidney the α-ENaC is responsive to aldosterone (44), whereas in the colon β and γ but not α-ENaC are induced by aldosterone (20, 45). In addition to induction of ENaC subunits the mRNA levels of the α- and β subunits of the Na⁺/K⁺-ATPase were increased upon aldosterone treatment in rat kidney epithelial cells (21, 22).
1.3. FKBP51 protein.

In our study we aimed to explore further the mechanism of aldosterone action by a search for new aldosterone-regulated genes. We identified FK506-binding protein 5 (FKBP51) as a major aldosterone responsive gene in distal colon. Since this protein has several functions directly or potentially related to aldosterone signaling it was selected for further study.

FKBP51 belongs to the immunophilin family of proteins. Immunophilins are so named because they bind the immunosuppressant drugs FK506. FKBP51 exhibits peptidyl-prolyl cis-trans isomerase (PPIase) activity that catalyzes the interconversion of prolyl–peptide bonds between the trans and cis states, with the trans state being energetically preferred in protein folding. FKBP51 has several functional domains. The N-terminal FK1 domain encompasses the PPIase function which is inhibited by the direct binding of FK506. The PPIase-like FK2 domain lacks measurable PPIase activity and cannot bind FK506. The C-terminal region contains a three- tetra-tripptide repeat (TPR) motifs involved in protein-protein interactions and serves as the site of interaction with the C-terminal sequence of Hsp90 that is bound to steroid receptors and other proteins (46).

1.3.1. Regulation of steroid receptor transcriptional activity.

FKBP51 is a co-chaperone and a component, along with HSP90 chaperone, of the GR/MR complexes. FKBP51 is a negative regulator of glucocorticoid and mineralocorticoid action and is replaced by the positive regulator, FK506-binding protein 52 (FKBP52) when these hormones bind to GR or MR, which renders the GR or MR complex active. The inhibitory effect of FKBP51 involves attenuation of steroid receptors affinity towards their ligands and inhibition of steroid receptor translocation into the nucleus (47-52). Although recently FKBP51 mediated inhibition of MR transcriptional activity was demonstrated, physiological relevance of this effect was not demonstrated and the effect of FKBP51 on aldosterone induced Na\(^+\) transport was not studied. We anticipated inhibitory effect of FKBP51 on Na\(^+\) transport but this was not its only possible role since FKBP51 is now emerging to be a multifunctional protein that, besides regulation of steroid receptors, has also a role in the activation of NF-κB pathway and is involved in additional cellular processes such as negative regulation of AKT (53). Such multi-functionality of FKBP51 is attributed to its HSP90 co-chaperone activity.
FKBP51 was shown to be induced by the synthetic glucocorticoid dexamethasone in lung and brain of murine models, in the human lymphoblastoid cell line IM-9, in human peripheral blood mononuclear cells, and in cultured murine podocytes (54-57). FKBP51 expression was also induced by progestins and androgens (58-60). It is considered to be a part of the negative feedback regulation of glucocorticoid and progestin signaling. While our study was in progress, small mineralocorticoid induced upregulation of FKBP51 was reported in endothelial and cardiomyocyte cell lines overexpressing MR (61,62). Yet, regulation of FKBP51 by physiological concentration of aldosterone in epithelia and relation to Na\textsuperscript{+} transport has not been reported before.

1.3.2. Activation of NF-κB pathway.
In addition to its role in GR/MR-mediated signaling it is also known that FKBP51 is an essential co-factor for the inhibitor of NF-κB kinase subunit alpha (IKKα) (63). IKK phosphorylates the inhibitor of NF-κB alpha (IκBα), resulting in proteosome-mediated degradation of IκBα and nuclear translocation and activation of NF-κB. Recently, aldosterone was shown to activate NF-κB and several either positive or negative effects of NF-kB on the transepithelial, aldosterone-induced Na\textsuperscript{+} transport have been reported (64-66). These findings raise the possibility that FKBP51 may be involved in regulation of Na\textsuperscript{+} transport or even in inflammatory effects induced by aldosterone by activating the NF-κB pathway.

1.3.3. Protective role of FKBP51 in oxidative stress.
Another recent finding is that FKBP51, besides its cytosolic localization, resides at significant amount in mitochondria (91). These authors also showed that oxidative stress causes translocation of FKBP51 from mitochondria into the nucleus and protection of cells against peroxide induced cytotoxicity. The underlying mechanism is not known. Yet, this finding suggests another link between aldosterone and FKBP51. Aldosterone is known to damage tissue partly by induction of oxidative stress (67). The well known aldosterone induced increase in Na\textsuperscript{+} transport evokes oxidative stress by largely elevating Na\textsuperscript{+}/K\textsuperscript{+}-ATPase activity and ATP hydrolysis (25). In addition aldosterone induces oxidative stress by several mechanisms such as increasing NADPH oxidase subunit expression, decreasing expression of glucose-6-phosphate dehydrogenase (G6PD) and
increasing expression of endothelin-1(67). It is possible that induction of FKBP51 is needed to protect cells against apoptosis during chronic aldosterone response.

1.4. Research objectives.

The aim of my thesis was to further explore the mechanism of aldosterone action by identifying new aldosterone-induced proteins. The starting point was a microarray screen of aldosterone responsive genes in rat distal colon. Unlike previous screens carried for convenience in cultured cells we have used native epithelium. The aldosterone-responsive epithelium selected was rat colon. Unlike kidney it is possible to obtain from this tissue a fairly homogenous population of aldosterone-responsive cells. It is also possible to treat colonic segment with aldosterone ex vivo and thereby expose native epithelium to well-defined concentrations of the hormone for a known period of time.

In our screen FKBP51 was identified as a major aldosterone-induced gene. In first part of my work I aimed to characterize regulation of FKBP51 in colon taking advantages of the ability to challenge colonic segments ex-vivo. The main questions addressed were:
- Is FKBP51 part of the early or late response to aldosterone?
- Which is (are) the receptor(s) involved? Is this response mediated by specific activation of MR, GR, or does it involve both receptors?
- Is FKBP51 induced by direct binding of activated steroid receptor to its promoter or does the response involve synthesis of a mediator protein?

In second part of my work I aimed to explore possible roles of FKBP51 in the known actions of aldosterone. For that purpose aldosterone responsive M1 cell line was produced.

The main questions addressed in this part were:
- Is FKBP51 involved in the regulation of aldosterone mediated increase in Na$^+$ transport?
- Does FKBP51 affect stability of MR?
- Does this protein have additional roles in hormone response such as protection of cells from aldosterone induced oxidative stress?
2. Materials and methods.

2.1. Materials.

Chemicals
Aldosterone, dexamethasone, cycloheximide, corticosterone, spironolactone, RU486, amiloride and carbenoxolone were purchased from Sigma. GSK650394 was obtained from TOCRIS bioscience, 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) was provided in cell proliferation kit (Biological Industries).

Antibodies
Anti-MR (rMR1-18 ID5) was obtained from Developmental Studies Hybridoma Bank, University of Iowa. Anti-GR (M20), anti p52/p100 (sc-7386), anti-p65 (sc-8008) and anti-FKBP51 (H-100) antibodies were purchased from SantaCruz. Anti-actin (cat. num. 69100) was purchased from MP Biomedicals, anti-fibrillarin (Ab4566) and anti-H2b (Ab45695) from Abcam and mouse and rabbit HRP-conjugated secondary antibodies were purchased from BIORAD.

Cell lines
The mouse cortical collecting duct cell line M1, was purchased from American Type Culture Collection (Mannasas, VA).

DNA constructs
The construct of rat MR subcloned into retroviral vector pLNCX was a kind gift by Dr. AnikóNáray-Fejes-Tóth (Dartmouth Medical School, Lebanon, NH USA). Human FKBP51 cDNA was a kind gift by Dr. Jonathan G. Scammell (University of South Alabama).
2.2. Methods.

2.2.1. Animal and tissue treatment.

In-vivo aldosterone treatment and RNA extraction from rat tissues.

Two groups of 11-13 weeks old Wistar rats were bilaterally adrenalectomized (ADX) and maintained for 4 days with an access to drinking water that contain 0.9% NaCl. Under these conditions the circulating level of adrenal steroids are lowered to zero but hyponatremia is prevented. One group was injected subcutaneously (sc) with a single dose of 50 µg/100g body weight aldosterone that was dissolved in phosphate buffered saline (PBS) containing 10% DMSO. In addition, these rats were implanted sc with osmotic minipumps which were filled with aldosterone dissolved in polyethylene glycol 300 (PEG300) at a concentration designed to achieve delivery rates of ~10µg/kg*hr. The other group was injected with the PBS containing 10% DMSO and implanted with osmotic minipumps delivering PEG300. Following 2-3 hours of aldosterone/vehicle administration rats were euthanized using CO₂ gas. Kidney and distal colon were isolated and total RNA was extracted with TRIzol reagent (MRC). Kidneys were dissected on an ice bed into cortex and medulla, and the different segments were homogenized in the TRIzol reagent using a Polytron (Kinematica Switzerland). The distal colon was cut open, rinsed in PBS, and immersed in the TRIzol reagent. The lysed epithelial cell layer was scraped off the connective tissue using a sterile glass slide, suspended in TRIzol reagent and homogenized. Modifications of the above protocol, used in some experiments, are described in the legends of the relevant figures. These include different concentrations of aldosterone and the use of non-adrenalectomized instead of ADX rats.

For protein quantification, tissue was excised from aldosterone treated and aldosterone depleted rats, dissected as above, and suspended in Buffer H composed of: 50mM β-glycerophosphate, pH 7.3, 1.5 mM EGTA, 1.0 mM EDTA, 1.0 mM dithiothreitol (DTT), 0.1 mM de-aerated sodium orthovanadate and protease inhibitors. Tissue was homogenized as above, centrifuged at 5000 rpm for 10 min and supernatants containing total protein were collected and analyzed by western blot.
**Ex-vivo aldosterone treatment of rat distal colon.**

For *ex vivo* incubation with aldosterone, the distal colon of adrenalectomized or non-adrenalectomized rats was cut longitudinally into two roughly equal segments, rinsed in PBS and incubated in 37 °C on gelatin sponge rafts soaked in PBS containing 5mM glucose, 5 mM sodium pyruvate with and without aldosterone and/or other reagents. Following the desired incubation period total RNA or protein was extracted as above.

**Isolating kidney collecting duct from transgenic mice.**

Two male mice expressing EGFP under the Hoxb7 promoter were kindly provided by Dr. Francesca Costantini (Department of Genetics and Development, Columbia University Medical Center, New York, USA). They were crossbred with wild type C57Bl females. The resulting offspring were genotyped by PCR analysis of DNA extracts from tail sections. F1 transgenic offspring were interbred to generate a colony of Hoxb7-EGFP mice.

Hoxb7-EGFP mice were euthanized, kidney was dissected and washed in cold PBS. Kidney cortex and medulla were minced on ice cold plate and pieces of kidney tissue were collected and incubated for 45 min at 37°C under shaking in a collagenase containing medium composed of: 136mM NaCl, 3mM KCl, 1mM K₂HPO₄, 1.2mM MgSO₄, 2mM CaCl₂, 4mM sodium lactate, 1mM sodium citrate, 6mM L-alanine, 5.5mM glucose and 0.5 mg/ml collagenase type IV. Every 10-15 min medium was replaced with fresh collagenase containing solution. Supernatants including kidney tubules were collected and centrifuged. The tubular pellet was suspended in ice cold DMEM-F12 medium containing 5% stripped serum and divided into small tissue culture plates placed on ice. Isolation of highly fluorescent tubular segments was performed under fluorescence microscope. Isolated tubular segments were incubated in tissue culture incubator in 5% stripped serum DMEM-F12 medium containing 10 nM aldosterone or vehicle for different time periods.

In other experiments mice had access for 6 days to drinking water containing 0.3% NaCl to lower plasma aldosterone, and then received a single injection of either 10 μg/kg aldosterone or vehicle. Two hours later mice were sacrificed and kidney cortex and medulla were sliced and treated with collagenase solution containing 10nM aldosterone.
or vehicle for 1 hour. Tubular segments were washed and collecting duct segments were isolated as above and incubated *ex-vivo* with 10nM aldosterone or vehicle for additional 2 hours, cells were centrifuged and RNA was extracted.

### 2.2.2. Microarray analysis of aldosterone induced proteins.

For global profiling of distal colon aldosterone-induced genes, adrenalectomized rats were treated as above for 2 hours, euthanized and colonic surface epithelial cells isolated by *in-vitro* incubation of inverted distal colons in 37°C in medium containing Ca^{2+} chelation reagents ±100 nM aldosterone for 40 min (68). Cells were centrifuged and total RNA isolated using TRIzol reagent. RNA quality was verified by Agilent 2100 Bioanalyzer (Agilent Technologies) and subjected to microarray analysis. Two GeneChip® Rat Exon 1.0 ST arrays (Affymetrix) were utilized to determine the colonocyte transcriptome profile of two untreated rats while additional two arrays were used to determine the transcriptome profile of two aldosterone treated rats. 1 µg of Total RNA extracted from colonocytes of a single rat was used per GeneChip® Rat Exon 1.0 ST array.

The microarray assay was done according to the Affymetrix technical manual. In brief, a double-stranded cDNA was synthesized from total RNA and an *in vitro* transcription reaction was done to produce biotin-labeled cRNA. The cRNA was then fragmentated and hybridized to the probe array. The hybridized probearray was stained with streptavidin phycoerythrin conjugate and scanned with GeneArray® Scanner to measure the amount of light emitted at 570 nm. This amount of emitted light is proportional to the bound target at each location on the probe array. Quality control assessment, which enables the identification of outlier arrays, problems during the labeling and/or hybridization, was carried out using the Expression Console software (Affymetrix). Statistical analysis of microarray data was performed using the Partek® Genomics Suite (PartekInc) software. To identify differentially expressed genes among the different treatments, a mixed model ANOVA analysis of variance was applied. Pairwise linear contrast was determined whenever required. To identify alternatively spliced exons, the Partek GS “Alt Splice ANOVA” pre-defined analysis was applied.
2.2.3. Co-immunoprecipitation of MR and GR.

Distal colon of ADX rats was rinsed in PBS and epithelial cell layer was scraped off the connective tissue into lysis buffer composed of: 20mM Tris-HCl pH 7.4, 150mM NaCl, 10mM MgCl₂, 2mM EDTA, 10% glycerol, 1% NP-40, 2.5 mM β-glycerophosphate, 1mM NaF and protease inhibitors. Cells were homogenized by passing through syringe, left on ice for 20 min and centrifuged at 14,000 rpm for 15 min to remove insoluble material. Aliquots of 200 μg total protein suspended in 300 μl PBS were pre-cleared on protein A beads for 1 hr. Anti-GR antibody was added to the supernatant and samples were rotated for 4 hr at 4°C. A control sample was treated identically with no added antibody. Next, 40 μl protein A beads were added and samples were rotated at 4°C overnight. Beads were washed 5 times with cold PBS and precipitated proteins were eluted by 5 min incubation in sample buffer at 95°C. Eluted proteins were resolved electrophoretically and blotted using anti-MR antibody.

2.2.4. Extraction of nuclear protein from colon tissue.

Distal colon was washed in saline containing: 20 mM Tris-HCl pH 7.0, 137 mM NaCl and 5 mM KCl. Cells were scraped off the connective tissue and homogenized in 9 ml buffer A composed of: 15 mM Tris-HCl pH 7.0, 60 mM KCl, 15 mM NaCl, 2 mM EDTA, 0.5 mM EGTA, 1 mM DTT, 0.15 mM spermine, 0.5 mM spermidine, 0.4 mM PMSF, 2 mM benzamidine, 0.25 M sucrose and protease inhibitors. The homogenate was mixed with 18 ml buffer B (buffer A plus 2.3 M sucrose), layered on top of 10 ml buffer C (buffer A with 1.8 M sucrose), and centrifuged at 25,000 rpm for 60 min at 4°C in a swinging bucket rotor. Pellet containing nuclei was solubilized in RIPA buffer composed of: 10 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% Triton X-100, 1% Na-deoxycholate, 0.1% SDS, left on ice for 15 min, briefly sonicated and nuclear protein lysate was then centrifuged at 14,000 rpm for 15 min to remove insoluble material.

2.2.5. Generation and measurements of cell culture models.

M1 cells were stably transfected with rat MR cDNA by the following protocol: MR containing retrovirus was generated by transient transfection of the construct into amphotropic Phoenix cells using Calcium Phosphate precipitation. Supernatant from amphotropic Phoenix cells producing retroviruses packaging were collected and passed through a 0.22-μm filter to remove cells and 1/1000 volume stock Polybrene solution was
added. Forty eight hours before infection, M1 cells at different concentrations were seeded onto 100-mm petri dishes. The medium from 50% confluent M1 cells was removed and replaced with 6 ml retrovirus supernatant and allowed to incubate for 6 h. This medium was removed and replaced with another 6 ml of fresh retrovirus medium. On the following day the above procedure was repeated twice. 48 hours later infected cells were split 1/10 and clones stably expressing MR were selected with G418 (GIBCO, 500 μg/ml). Expression of MR receptor was verified by western blotting using a monoclonal anti-MR antibody. These cell clones are referred to as M1-MR cells.

M1-MR cells were transfected with either FKBP51 cDNA subcloned into pIRES-puro vector or with a commercial FKBP51 shRNA construct (Sigma). Transfections were carried out using JET-PEI (Polyplus-transfection Inc.). Antibiotic resistant clones were selected with puromycin, further cultivated and tested for FKBP51 expression using anti-FKBP51 antibody.

For transepithelial electrical measurements M1 cells were seeded onto 12mm Millicell permeable membranes (Millipore-PCF) at a density of 5.6x10⁴-1.7 x 10⁵ cells per filter. After seeding, cells were bathed in complete PC1 medium (BioWhit-taker, Walkersville, MD) with 5% FBS for 5-8 days until cell monolayers developed transepithelial resistance >1 kOhm*cm² and stable short-circuit current values. To determine the effects of aldosterone, confluent cell monolayers were maintained overnight in steroid-free (stripped) medium (DMEM/F12 + 5% FBS that was passed six times on charcoal/15 mM Hepes/2mM glutamine/antibiotics), and then treated with 10 nM aldosterone for different periods of time. Transepithelial voltage (TEV) and resistance (TER) were determined on the PCF membranes with an epithelial volt/ohm-meter (World Precision Instruments, Sarasota, FL), and the short-circuit current (Isc) was calculated as TEV/ TER. It was previously demonstrated that in M1 cells Isc mainly represents transepithelial Na⁺ current (69). This was verified by applying 10 μM of the ENaC blocker amiloride to the apical side, which resulted in an almost total elimination of the lumen negative TEV and the associated Isc.

For RNA extraction, cells were seeded on 24mm Costar polyester membranes and cultivated as above. Confluent cell monolayers were maintained overnight in steroid-free
medium (DMEM/F12 + 5% FBS that was passed six times on charcoal/15 mM Hepes/2mM glutamine/antibiotics), and then treated with 10-100 nM aldosterone for different periods of time. Cells were scraped off the polyester membranes into RLT buffer and total RNA was prepared using RNeasy kit (QIAGEN).

2.2.6. Cell proliferation under oxidative stress.
Cell proliferation rate was quantified by reduction of XTT. M1-MR cells (1.5-3x10^4 cells) were seeded in 96 well plate in a phenol red free DMEM/F12 medium containing 5% FBS. In the next day cells were incubated with 500 μM H₂O₂ for 5 hours and then 40μl XTT reaction solution was added into the medium and cells were incubated for additional 2 hours. Absorbance was measured at 450 nM and non-specific background, measured at 650nM, was subtracted.

2.2.7. Extraction of nuclear protein from cultured cells.
M1 cells grown in 10 cm plate were washed with PBS, scrapped into cold PBS and centrifuged for 4 min at 2000 rpm. The pellet was gently re-suspended in 200 μl of lysis buffer compose of: 10 mM Tris-HCl pH 7.5, 100 mM NaCl, 1.5 mM MgCl₂, 0.5% NP-40 and protease inhibitors. The lysate was vortexed for 10 seconds and centrifuged for 3 min at 2500 rpm. Supernatant containing cytosolic fraction was removed and the nuclear pellet was washed with 100 μl lysis buffer and centrifuged again. Nuclei were solubilized on ice in RIPA buffer for 15 min, briefly sonicated and the nuclear protein lysate was centrifuged at 14,000 rpm for 15 min to remove insoluble material.

2.2.8. Real-Time Relative RT-PCR.
Total RNA was made free of residual genomic DNA contamination using the DNaseI treatment and removal kit (Ambion). Purified RNA was reverse-transcribed with Verso reverse transcriptase (Thermo scientific) and PCR reactions were set up using the POWER SYBR Green PCR kit (Applied Biosystems) according to the manufacturer's instructions. Fluorescence changes during real-time PCR were measured with an AB 7300 real-time PCR system (Applied Biosystems).
The following primers were used for PCR:

<table>
<thead>
<tr>
<th>Protein</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
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<tbody>
<tr>
<td>β-actin</td>
<td>5′-CACTGCCGCATCCTCTCC-3′</td>
<td>5′-GATGCCACAGGATCCATCC-3′</td>
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<tr>
<td>GAPDH</td>
<td>5′-CTGCACCACCAAACCTGCTC-3′</td>
<td>5′-CATGGACTGATGTCATGAGC-3′</td>
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<td>5′-GCTTCCAGAATCACAGAGAC-3′</td>
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<tr>
<td>Arginase2</td>
<td>5′-ACGCTCTTGCCAGAGGCTT-3′</td>
<td>5′-GTCAGACTGAGGTCTTTAG-3′</td>
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<tr>
<td>AngII-AVP receptor</td>
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<tr>
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<td>p23</td>
<td>5′-GAGGATGACTCAGATGGAAGAC-3′</td>
<td>5′-CTTTCGATACATCATCTGC-3′</td>
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</table>

The results obtained for different transcripts were normalized to the endogenous controls β-actin or GAPDH.

2.2.9. **Fluorescent microscopy.**

Kidneys of transgenic Hoxb7-EGFP and wild type mice were fixed with 4% paraformaldehyde for 6 hours in 4°C. Fixed tissue was pretreated with 30% sucrose in PBS overnight. Kidney samples were embedded in Tissue-Tek OCT compound (Sakura Finetechical), and cryosections of 10 µm thickness were cut with a cryostat and mounted on glass slides. GFP fluorescence was visualized microscopically.
2.2.10. *Western blot analysis.*

Equal amounts (30 μg) of protein were resolved by SDS-PAGE electrophoresis in 7.5% acrylamide gels and transferred onto PVDF (Millipore) using a semi-dry blotting apparatus. Membranes were blocked with Phosphate-buffered saline containing 0.1% Tween20 (PBS-T) and 5% dry milk for 1 h at room temperature. The blot was cut into several regions and incubated with different primary antibodies in PBS-T containing 1% BSA for overnight at 4°C. Membranes were washed 3 times in PBS-T and then incubated for 1 h at room temperature with an appropriate HRP-conjugated secondary antibody in PBS-T containing 5% dry milk. Membranes were then washed 3 times in PBS-T and protein expression was assayed by enhanced chemiluminescence and quantified using ImageQuant LAS 4000 mini (General Electric).
3. Results


3.1.1. Microarray analysis of aldosterone induced genes in distal colon.

A microarray analysis has been performed on distal colon epithelial mRNA isolated from untreated and aldosterone stimulated ADX rats (2 h \textit{in vivo} plus 0.6 h \textit{ex-vivo}). The screen identified 71 significantly (>1.9-fold) regulated mRNAs. Some of these genes were clustered into the following functional groups: ion transport, intracellular trafficking, lipid metabolism, steroid metabolism, apoptosis and genes involved in the defense response. Among the differentially expressed genes we were able to identify the majority of the previously known aldosterone up-regulated genes. These include - SGK1, \( \gamma \)ENaC and GILZ (Fig. 1A). This fact emphasizes that colon tissue is a legitimate and convenient model to explore the mechanism of aldosterone action.

Fig. 1B demonstrates major new aldosterone induced genes that have a potential to be involved in aldosterone mediated transport. Among these, FKBP51 (FK506-binding protein 5), was the most strongly induced gene, up-regulated by 32 fold.

\textbf{Fig.1 Microarray assay of colonic aldosterone regulated genes.}

For microarray analysis 2 ADX rats were treated with aldosterone as described in the methods and additional 2 ADX rats received vehicle. Surface colonocytes were isolated \textit{ex-vivo} for 40 min in the presence or absence of aldosterone. Colonocyte total RNA was prepared and used for GeneChip \textsuperscript{®} Rat Exon 1.0 ST array profiling. The figure presents microarray results for previously known aldosterone induced genes (A) and for new aldosterone regulated genes (B). Statistical significance was calculated using ANOVA analysis of variance.

<table>
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<tr>
<th>Microarray results</th>
<th>B. Microarray results</th>
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<tr>
<td>Previously known aldosterone regulated genes</td>
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<tr>
<td>Sgk1</td>
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</tr>
<tr>
<td>( \gamma )ENaC</td>
<td>34</td>
</tr>
<tr>
<td>GILZ</td>
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<table>
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<th>New aldosterone regulated genes</th>
<th>mRNA fold change</th>
<th>p-value</th>
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<td>Arginase2</td>
<td>9</td>
<td>&lt;0.04</td>
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<td>Cdc37</td>
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<td>&lt;0.05</td>
</tr>
<tr>
<td>Mucolipin2</td>
<td>5</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>PLK2</td>
<td>0.29</td>
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<tr>
<td>AngII/AVP receptor</td>
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<td>&lt;0.03</td>
</tr>
<tr>
<td>Kelch5</td>
<td>5.6</td>
<td>&lt;0.03</td>
</tr>
</tbody>
</table>
3.1.2. *In-vivo aldosterone mediated induction of FKBP51.*

Microarray identified aldosterone-regulated genes were validated by quantitative PCR using several independent colonic RNA preparation (Fig. 2A). FKBP51, Arginase2 and Mucolipin2 were three of the most strongly and significantly induced genes up-regulated by 33, 3.5 and 3.2-fold respectively. Clearly FKBP51 appeared to be dramatically regulated and was by far the most aldosterone responsive gene identified in our study. The colonic epithelium has been selected for the above analysis for a number of reasons discussed under introduction. Yet, since the kidney is the major site for aldosterone action we wanted to confirm some of the above data in kidney preparations as well. Quantitative PCR analysis confirmed regulation of FKBP51 and PLK2 in kidney medulla although a considerably milder induction of FKBP51 was noted (Fig. 2B). FKBP51 mRNA was induced 4.3-fold while PLK2 was reduced 0.59-fold. Several other mineralocorticoid regulated genes validated in colon appeared not to be induced by aldosterone in kidney.

**Fig.2: RT-PCR validation of microarray results: in-vivo regulation of FKBP51.**

(A) ADX rats were treated with aldosterone or vehicle for 2.5 hours as described under methods. RNA was prepared from scrapped colonic cells and analyzed by RT-PCR. Bars are means ±SEM, n=3-7 rats. (B) ADX rats were treated for 2 hours by injecting 25 µg/100g body weight aldosterone or diluent and implanting osmotic pump which deliver ~5µg/kg*hr of aldosterone or diluent. RNA was prepared from dissected kidney medulla and analyzed by RT-PCR. Bars are means ±SEM, n=7-11 rats. * P<0.05 , ** P< 0.005, *** p<0.0005 calculated using unpaired t-test.
3.1.3. Ex-vivo aldosterone mediated induction of FKBP51.

The experiment of Fig. 2 demonstrated induction of FKBP51 in colon and kidney medulla. This effect however required in vivo stimulation by a poorly defined dose of aldosterone. In order to characterize aldosterone regulation of FKBP51 by defined aldosterone concentration we used ex-vivo treatment of colonic and kidney preparations.

Due to the large air exposed surface of distal colon, this tissue unlike kidney, can be maintained ex-vivo for at least several hours with little ischemic damage (70). This enables to study regulation of RNA and proteins in native tissue, exposed to known concentrations of the hormone for a well-defined time. Accordingly, RNA was extracted from distal colons of ADX rats that were incubated in medium containing 30 nM aldosterone or vehicle for 2.5 hours. This concentration corresponds to the maximal plasma aldosterone level achieved under low Na⁺ diet (71). Figure 3A demonstrates the up-regulation of FKBP51, Arginase2 and Hypothetical protein (NM_001039002) mRNAs by 15.8, 2.3 and 2.2 folds, respectively. The other genes- cdc37, Kelch5, mucolipin2, PLK2 and AngII/AVP receptor were not shown to be increased by 30 nM aldosterone ex-vivo probably because they are stimulated by supraphysiological doses of mineralocorticoid through GR which implies that they may be regulated by glucocorticoids in physiological responses to stress.

The above data is the first evidence for induction of FKBP51 by physiological concentrations of aldosterone. Since FKBP51 was the gene to be up-regulated most strongly both in distal colon and in kidney and is clearly related to the hormone action, we have selected it for further characterization. Mature MR and GR complexes consist of an hsp90 dimer, p23 co-chaperone, possibly hsp70, and one of the tetratricopeptide repeat (TPR)-containing proteins, such as the FKBP51 or FKBP52 (72). In light of the above data it was of interest to explore whether FKBP52 and p23 are too regulated by aldosterone. However, neither FKBP52 nor p23 mRNA appeared to be regulated in colon by ex-vivo treatment with aldosterone (Fig.3B).
Distal colons of ADX rats were cut longitudinally into two equal halves and incubated on gelatin sponge rafts soaked with medium containing 30 nM aldosterone or diluent for 2.5 hours. A. Colonic cells were scraped off the connective tissue, RNA extracted and analyzed RT-PCR. Bars are means ±SEM, n=3 rats. * P<0.05 , ** P< 0.005, calculated using paired t-test. (B) Distal colon segments of ADX rats were incubated as above in medium containing 10 nM aldosterone for 5 hours. RNA was analyzed by RT-PCR using FKBP52 and p23 specific primers. Bars are means ±SEM, n=3 rats.

The in vivo induction of FKBP51 in kidney depicted in Fig. 2B was measured in crude medulla preparation in which fraction of the RNA is derived from aldosterone responsive cells. In order to obtain a preparation of higher purity and also achieve a more controlled exposure to aldosterone, we aimed to establish a protocol for isolating fresh aldosterone responsive collecting duct tubules from kidney and either treat them with aldosterone ex-vivo or grow primary cultures of principal cells. Experiments were done using a colony of Hoxb7-EGFP transgenic mice in which EGFP is expressed under the regulation of Hoxb7 promoter that is activated specifically in the collecting duct (Fig. 4A) (73).

Kidney cortex and medulla were treated with collagenase and collecting tubular fragments were isolated under fluorescence microscope. Cells were dissociated from the selected tubules, seeded in 48 well plates and grown till confluency in primary culture medium. However, after several days in culture cells became insensitive to aldosterone assessed by the lack of Sgk-1 activation. An alternative approach attempted was to isolate tubules and incubate them in-vitro at 37°C ±10 nM aldosterone for 2 hours. This treatment caused 1.2 folds induction of FKBP51 RNA while Sgk-1, a marker for the early effect of the hormone, was induced by 1.76 folds (Fig.4B). In attempt to prolong the
stimulation period, we injected mice with 10 μg/kg aldosterone for 2 hours and then isolated collecting duct fragments in the presence of aldosterone for 1 hr and incubated them in-vitro with 10 nM aldosterone for another 2 hours. This protocol too failed to evoke a substantial up-regulation of FKBP51 mRNA while Sgk-1 was induced by 2 folds (Fig. 4C). Thus, treating isolated tubules with 10 nM aldosterone for 2-5 hours does not induce the same massive increase in FKBP51 mRNA seen in colon. It should be noted that a different response of colon and kidney has been observed for other aldosterone induced genes as well i.e. α vs, β and γ ENaC or CHIF (20, 44, 45, 74).

Fig.4: Ex-vivo aldosterone mediated regulation of FKBP51 in kidney collecting duct.

(A) Cryosections of Hoxb7-EGFP transgenic mice visualized by direct fluorescence microscopy. (B) Fluorescent collecting duct tubular segments were isolated from suspension of kidney cortex and medulla tubuli of Hoxb7-EGFP mouse and incubated ex-vivo for 2 hours with 10 nM aldosterone or vehicle. RNA was isolated and assayed for the abundance of FKBP51 and SGK1 mRNA’s by RT-PCR. (C) Mice received a single injection of 10 μg/kg aldosterone or vehicle. Two hours later mice were sacrificed and kidney cortex and medulla collecting duct tubular segments were isolated as above in the presence of aldosterone for 1 hr and treated ex-vivo with 10 nM aldosterone or vehicle for additional 2 hours. RNA was isolated and assayed for the abundance of FKBP51 and SGK1 by RT-PCR.
3.2. Characterization of aldosterone induced regulation of FKBP51.

3.2.1. Regulation of the FKBP51 protein by aldosterone.

Next, we have tested whether the observed increase in FKBP51 mRNA is accompanied by a similar increase at the protein level. Short term treatments of rats with aldosterone i.e. $\leq$ 5 hours had very little or no effect on the FKBP51 protein (not shown). However, a prolonged 24 hours treatment with aldosterone delivered through osmotic minipumps, resulted in a large increase of the abundance of FKBP51 protein in the colon (Fig.5A). This increase was not matched by up-regulation of FKBP51 protein in kidney medulla (Fig.5B). The apparent lack of protein induction in kidney medulla is in agreement with a ~7 time smaller induction of FKBP51 mRNA in the kidney (cf. compare the data of Figures 2A and B) and no significant induction in isolated collecting duct.

**Fig.5: Regulation of FKBP51 protein by aldosterone.**

ADX rats were treated with aldosterone by implanting them with osmotic mini pump that delivers ~10µg aldosterone/kg/hr. After 24 hours proteins were extracted from distal colon (A) and kidney medulla (B) and analyzed on western blot using anti-FKBP51 antibody. Each lane includes the same amount of total protein extracted from colon or medulla of a different rat.

The antibody used in these experiments is directed to the C-tail of FKBP51 and therefore should identify all five alternatively spliced forms of the protein (75). The fact that only the $>50$kDa species was detected indicates that colonocytes express only the full length FKBP51 with two peptidylprolyl isomerase domains and three tetra-tricopeptide repeats.
3.2.2. Time course and dose response of FKBP51 induction.

In the next set of experiments dose-response and time course of the aldosterone-induced expression of FKBP51 were explored. These were done by ex vivo treatment of distal colon isolated from ADX rats with defined concentration of the hormone for different periods of time. Figure 5 depicts RT-PCR analysis of FKBP51 using total colonic RNA extracted after different manipulations. Treating colonic tissue by 10 nM aldosterone, a concentration that is well within the Na\(^+\) diet-induced variations of plasma aldosterone, evoked a gradual increase in the abundance of FKBP51 mRNA (Fig. 6). While a significant increase in FKBP51 mRNA is apparent already 2.5 hours after applying the hormone (5.9±0.6 fold  P<0.003), most of the increase (45±9.6 folds  P<0.02) develops afterwards. Taken together with the fact that protein induction lags behind the increase in mRNA, the above data indicates that FKBP51 is involved in the “late” or chronic response to the hormone that correlates in time with the inductions of ENaC subunits and the Na\(^+\)/K\(^+\) ATPase but lags behind the Sgk-1 induced activation of channels (25, 70, 76).

**Fig. 6: Time course of the ex-vivo induction of FKBP51**

Distal colons of ADX rats were cut longitudinally to two equal halves and incubated ex-vivo for 5 hours. One hemi colon received 10 nM aldosterone while the other served as control and received diluent. The hormone was added at different times after initiating the ex vivo incubation, enabling different hormone incubation times but the same ex vivo incubation period. RNA was extracted and analyzed by RT-PCR for the abundance of FKBP51 mRNA. 3 colonic segments from different rats were used for each time point. Data points are Means ±SEM.

Fig. 7 depicts a dose-response curve of the increase in FKPB51 mRNA evoked by 1-300 nM aldosterone. Lineweaver–Burk plot of the mRNA induced at 3-300 nM aldosterone was characterized by Km of 22 nM and V\(_{\text{max}}\) of 98 fold increase. This Km is higher than Kd of aldosterone for MR and lower than Kd of aldosterone for GR (26, 77-81). Thus, it appears that induction of FKBP51 by aldosterone in colon does not follow a simple
model of populating MR or GR alone and suggests more complex scheme which involves both receptors.

**Fig. 7: Kinetic analysis of the Aldosterone dose-response relationships.**

Colonic tissues from ADX rats were incubated as described above for 5 hours with the indicated concentrations of aldosterone. Means ±SEM of data from 2-7 rats are depicted. **Insert:** Lineweaver-Burk analysis of the data corresponding to concentrations of 3-300 nM aldosterone.

**3.2.3. Regulation of FKBP51 by glucocorticoids.**

Since most of the increase of FKBP51 mRNA was evoked by aldosterone concentrations likely to populate GR we tested the ability of glucocorticoids to produce the same response. Incubating tissue with 150 nM corticosterone, the main glucocorticoid in rodents, had no apparent effect on the abundance of FKBP51 mRNA nor did it inhibit the response to 10nM aldosterone (Fig. 8A). On the other hand, similar amount of the synthetic glucocorticoid and mineralocorticoid agonist dexamethasone increased FKBP51 mRNA by 74±8.2 folds. The difference between the two could in principle be accounted for by different sensitivity to 11-β-hydroxysteroid dehydrogenase-2 (11βHSD-2). This enzyme catalyzes the conversion of glucocorticoids to the corresponding 11-dehydroxy metabolites. In the case of natural occurring glucocorticoids such as
corticosterone, the product 11-dehydrocorticosterone is inactive as MR or GR agonist. 11-dehydroxy dexamethasone on the other hand, can still function as GR agonist (82, 83). To test this hypothesis we examined effects of the 11βHSD-2 inhibitor carbenoxolone (CBX)(84). Incubating colonic fragment with 10 µM CBX evoked a significant but very limited induction of FKBP51 by corticosterone (7.2 ± 1.8 folds P<0.02) (Fig. 8A). Thus, high 11βHSD-2 activity cannot account for the inability of corticosterone to induce FKBP51. Whatever the reason for ineffectiveness of corticosterone might be, the fact that FKBP51 is not induced by corticosterone in colon suggests that this gene is not regulated by native glucocorticoids in colon in contrast to what was shown in lung and brain tissues of murine models (54, 55). However, it is possible that under stress conditions higher concentration of corticosterone could cause up-regulation of this gene in colon as well.

An issue which relates to the corticosteroid specificity of the response is the fact that the above experiments were all carried out in rats that underwent bilateral adrenalectomy and were therefore adapted to near zero level of adrenal steroids. This may give rise to two non-physiological behaviors. (1) That in distal colon too FKBP51 is normally stimulated by glucocorticoids and the response to aldosterone is due to the fact that the tissue was adopted to “zero glucocorticoid” status. (2) that the response of aldosterone is affected by the lack of glucocorticoid metabolites. Several investigators have reported that effects of aldosterone on sodium retention are inhibited by the presence of glucocorticoids or their 11-dehydro derivatives. It was therefore proposed that 11βHSD-2 products can bind MR and act as competitive inhibitors of aldosterone (28, 85). The fact that corticosterone had no significant effect on the aldosterone-induced increase in FKBP51 mRNA argues against such possibility. Nevertheless we also examined elevation of FKBP51 mRNA in non-adrenalectomized (non-ADX) rats. As shown in Fig. 8B distal colon from non-ADX rats too exhibited a highly significant aldosterone-induced induction of FKBP51 when incubated ex-vivo. The response appeared to be smaller than the one obtained for ADX rats (8.6 folds in non-ADX vs. 48 folds in ADX rats) but yet profound and larger than other previously reported mRNAs (29-35). This finding rules out the above possible non physiological responses.
3.2.4. Steroid receptors involved in aldosterone mediated FKBP51 induction.

To dissect the relative contribution of the GR and MR in FKBP51 induction we assessed the effects of receptor specific antagonists. The effects of antagonists were tested at 10 nM and 100 nM aldosterone that should fully occupy either MR alone or both MR and GR, respectively (Fig. 9A). 10 nM aldosterone, which should fully occupy MR with very limited GR occupancy induced 26±2.6 folds increase in FKBP51 while 100 nM aldosterone, which fully occupy both receptors, increased FKBP51 by 59±4.5 folds. The fact that 100nM aldosterone produced more than 2 fold higher response than 10 nM suggests involvement of GR. Surprisingly, induction of FKBP51 by either 100 nM or 10 nM aldosterone, was fully blocked by the MR specific antagonist spironolactone. Also unexpected was the fact that the GR antagonist RU486 was without effect on the response to high doses of aldosterone. RU486 did inhibit induction of FKBP51 by dexamethasone (Fig.9B), excluding a technical problem as the reason for its ineffectiveness in aldosterone treated tissue.
One mechanism that may account for the above observations is a response mediated by a MR-GR heterodimer, reported before in other systems. In this case the lack of inhibitory effect of RU486 could be explained by the fact that it does not prevent translocation of GR into the nucleus where GR might associate functional MR and form transcriptionally active heterodimer. Support for this hypothesis was provided by the surprising stimulatory effect of RU486 on the induction of FKBP51 by low doses (3nM) of aldosterone (Fig.9C). This issue as well as other potential mechanisms are further elaborated in the Discussion. Yet, it prompted examination of the existence of MR-GR dimers in colonocytes. This has been tested by immunoprecipitation of GR from colonocytes extracts and blotting the immune pellet for MR. Fig.9D shows that MR is indeed co-precipitated with GR indicating existence of MR-GR dimers in colon and supporting the hypothesis that FKBP51 could be induced by MR-GR dimer. The heterodimers exist in control colons and in colons incubated with aldosterone. Surprisingly, Fig.9D also shows that mineralocorticoid stimulation of distal colon reduces the level of MR-GR dimers. This could be explained by aldosterone mediated downregulation of GR expression that was demonstrated previously (86).

**Fig.9 : Effects of MR and GR antagonists on the response to aldosterone.**

(A) Colonic tissues from ADX rats were incubated for 5 hours with 10 nM aldosterone ±1µM spironolactone or 1µM RU486. Another group of colonic fragments was incubated with 100nM aldosterone ±10µM spironolactone or 10µM RU486. 3-4 colonic segments were used for each treatment. Bars are Means ±SEM (B) Colonic tissues of ADX rats were incubated for 5 hours with 100 nM dexamethasone ±10µM RU486. 3-4 colons were used in each group. Bars are Means ±SEM. (C) Colonic tissue fragments from ADX rats were incubated for 5 hours with 3 nM aldosterone ±10µM RU486. 4 colons were used in each group. Bars are Means ±SEM. (D) Distal colons of 2 ADX rats were cut into two halves that were incubated with 100 nM aldosterone or vehicle for 2.5 hours. Protein was extracted and equal amounts of protein were subjected to immunoprecipitation using anti-GR antibody or served as negative control and incubated with protein A beads only. Co-immunoprecipitated MR was analyzed by western blot with anti MR. The figure depicts representative blot of experiments repeated three times. Shown is the immunoprecipitated MR (IP) next to the sample pulled down by beads only (B) and a blot of the pre-immunoprecipitated lysate (TOTAL).
3.2.5. Primary vs. secondary actions of aldosterone on FKBP51.

Aldosterone elicits its effect by activating translocation of MR and/or GR to the nucleus and their subsequent binding to hormone responsive elements in the promoters of aldosterone regulated genes, leading to alteration of their expression. Some of these aldosterone-regulated genes could be transcription factors giving rise to secondary aldosterone-dependent responses. To test whether the induction of FKBP51 is a direct response mediated by the binding of activated receptors to its promoter, we examined the effect of protein synthesis inhibitors on the induction of FKBP51 mRNA. Cycloheximide (CHX), a well established blocker of translational elongation in eukaryotes (87), inhibited 65% of the induction of FKBP51 mRNA by aldosterone (p<0.0008) (Fig.10A). This effect of CHX was somewhat surprising since previously published data indicate that CHX does not inhibit induction of FKBP51 mRNA by glucocorticoids (57). Therefore we have also tested for effect of CHX on the dexamethasone induced induction of FKBP51 in colon and aimed to dissect the response to “MR-dependent” and “pure GR” actions. This was achieved by the addition of either spironolactone (to block MR response) or RU486 (to block GR response). Indeed, the effect of CHX on the response to dexamethasone was small and statistically insignificant (p>0.09) (Fig.10A). However when the “MR-dependent” component of the response was measured by including RU486, CHX inhibited 66% of the response (p<0.0003) (Fig.10A). Thus, the current MR dependent response evoked either by aldosterone or by dexamethasone is inhibited by CHX and therefore secondary to the induction of other protein. The previously described GR specific effect on the other hand, is a primarily one.

A possible mediator of FKBP51 induction could be the aldosterone induced SGK1, shown before to activate transcription of αENaC, a secondary aldosterone induced protein (88). To test this option we analyzed the effect of the recently available Sgk-1 inhibitor GSK650394 on the aldosterone-induced up-regulation of FKBP51 (89). No effect of GSK650394 on the response to aldosterone was noted, arguing against involvement of Sgk-1 in FKBP51 transcription (Fig.10B).
Fig. 10: The effect of protein synthesis inhibitors on corticosteroid mediated regulation of FKBP51 mRNA.

(A) Distal colon segments from non-adrenalectomized rats were incubated ex-vivo for 5 hours in the presence of the following four combinations of steroids: (1) 10nM aldosterone ± 1μM cycloheximide (CHX). (2) 100nM dexamethasone ± 1μM CHX. (3) 100nM dexamethasone + 10μM spironolactone (GR specific effect) ± 1μM CHX. (4) 100nM dexamethasone + 10μM RU486 (MR specific effect) ±1μM CHX. 3-8 colon segments were used in each group. Probabilities were calculated by paired t-test. (B) Colonic segments were incubated for 5 hours in medium containing 10nM aldosterone alone or in combination with 3-10 μM of the Sgk-1 inhibitor, GSK650394. 4-6 colon segments were used in each group. Bars are Means ±SEM.

A.

B.
3.3. Functional effects of FKBP51.

The next issue addressed is the possible role of FKBP51 in the hormonal response and the reason it is so strongly induced by aldosterone. In principle this could involve one or more of the above described MR-dependent and MR-independent functions of this protein. One possibility is that FKBP51 binds to MR and either inhibits its function or stabilizes its structure. As elaborated in the Discussion, this may be required for termination, synchronization or stabilization of the hormonal response. A second mechanism considered is the possible effect of FKBP51 on Na\(^+\) transport via activation of NF-kB pathway. Another suggested mechanism is protection of cells from aldosterone induced oxidative stress. These putative mechanisms are not mutually exclusive. Thus, it is possible that FKBP51 is involved in more than one of them and depending on concentration, time and location has different and even opposite effects. Accessing the above possibilities requires system in which effects of aldosterone on Na\(^+\) transport can be monitored at different levels of FKBP51, as well as measurements of MR and NF-kB abundance and location. Such experiments are described in the following sections.

3.3.1. Establishment of aldosterone responsive M1-MR cells.

To address the above possibilities it was essential to establish a cell culture system in which effects of varying levels of FKBP51 on the aldosterone-induced response can be studied. Intestine derived cell lines such as HT29 and Caco-2 do not form tight epithelia and do not manifest sizeable transepithelial Na\(^+\) transport. Two collecting duct derived aldosterone responsive cell lines have been reported, M1 and mCCD(c1) (26, 90). mCCD(c1) is not freely available while M1 can be purchased from ATCC. These cells have lost the MR receptor during immortalization. Therefore the first aim was to transfet them with MR and establish an aldosterone-responsive cell line. Cell clones which stably express MR (M1-MR) were isolated (Fig.11A). The ability of these cells to manifest physiological responses to aldosterone has been tested by two criteria: (a) ability to induce Sgk-1 and (b) an increase of active Na\(^+\) transport measured as an amiloride-blockable transepithelial short circuit electrical current. The M1-MR cells responded to 10 nM aldosterone by an increase in Sgk-1 (Fig.11B). When cultivated in filter bottom cups in complete medium both M1 and M1-MR cells formed tight epithelium characterized by transepithelial resistance of >1 kOhm*cm\(^2\) and significant transepithelial electrical potential of 40-60 mV. However, aldosterone significantly increased
transepithelial Na\(^+\) transport (Isc) only in M1-MR cells but not in M1 cells. Therefore MR overexpression is required for aldosterone mediated increase in Na\(^+\) transport in M1 cells (Fig.11C).

**Fig.11: Functional characterization of M1-MR cells.**

(A) M1 cells were infected with rat mineralocorticoid receptor subcloned into the adenoviral vector pLNCX. Several antibiotic resistant colonies were selected and assayed for the expression of MR. Western blots of non transfected (M1) and transfected (M1-MR) with anti-MR antibody is depicted. (B) M1-MR cells were seeded on permeable support and grown in a complete medium. After reaching confluency, cells were maintained in steroid-free medium overnight and then treated with 10 nM aldosterone for different periods of time. Cells were scrapped off the membrane, RNA prepared and analyzed for the abundance of Sgk-1 mRNA by RT-PCR. (C) M1 and M1-MR cells were cultivated in complete medium in filter bottom cups and the transepithelial electrical voltage (TEV) and resistance (TER) were monitored daily. After TER reached value of >1 kOhm*cm\(^2\) and TEV reached constant value, cells were transferred to a steroid free medium. Following 24 hr. cells were challenged by 10 nM aldosterone and TEV and TER were recorded over time. The experiment was terminated by adding amiloride (10\(\mu\)M) to the luminal side. The figure depicts short circuit currents (Isc) calculated as TEV/TER which in this system is equivalent to the transepithelial Na\(^+\) flux. Shown is a representative experiment performed in duplicates. Bars are Means ±STDEV.
Independently we have also shown that treating M1-MR cells with 100 nM aldosterone elevates FKBP51 mRNA and the magnitude of this effect depends on MR expression (Fig. 12). Thus, even though the response is small relative to native colonic epithelium, M1-MR cells are a legitimate system to study the effects of FKBP51 on aldosterone mediated response. Moreover, in these cells too induction of FKBP51 is mediated by MR but achieved only at high doses of the hormone.

**Fig.12: Regulation of FKBP51 in M1-MR cells.**

M1 cells that do or do not express MR were grown in a complete medium. After reaching confluency, cells were maintained in steroid-free medium overnight, and then treated with 100 nM aldosterone for 5 hours. FKBP51 mRNA was quantified and normalized to the value in steroid depleted samples. Means ±SEM of 3 experiments are depicted. * P<0.03, **P<0.0.0003 calculated by paired t-test.

![Graph showing regulation of FKBP51 in M1-MR cells.](image)

3.3.2. The effect of FKBP51 on MR level.

To study the role of FKBP51 in the response to aldosterone M1-MR cells were stably transfected with FKBP51 (M1-MR-FKBP51). Three clones expressing high amount of FKBP51 are shown in Fig. 13. In two of them the expression of FKBP51 elevated MR levels by ~1.8-2.8 fold, suggesting that FKBP51 has a tendency to increase MR level. The opposite approach, namely silencing endogenous FKBP51 by transfection of appropriate shRNA has been attempted as well. Two such clones, denoted 6a1 and 9b6, are shown in Fig 13C while Fig.13D quantifies MR and FKBP51 levels in 11 different clones. A clear outcome is that the decrease in FKBP51 level is also associated with MR degradation. Variable and in most cases substantial decrease of MR were obtained in the FKBP51 silenced clones. This is with exception of a single clone that for unknown reason manifested large increase.
To study the effect of FKBP51 knockdown on aldosterone induced Na\(^+\) transport we selected clones 9A4L and 9A6L that exhibit 95% and 91% reduction of FKBP51 while having only a small inhibitory effect on MR level (13% and 34%, respectively) (Fig.13D).

**Fig.13. Manipulating FKBP51 protein level in M1-MR cells and its effect on MR expression.**

(A) M1-MR cells were stably transfected with human FKBP51. M1-MR and three M1-MR-FKBP51 cell clones denoted cl.1, cl.2 and cl.3 were grown in complete medium and then transferred to stripped serum containing medium. On the following proteins were isolated and analyzed for the expression of MR and FKBP51 by western blot. (B) Densitometric analysis of western blot in (A). (C) Endogenous FKBP51 was silenced in M1-MR cells by stable transfection of FKBP51-shRNA. M1-MR and 11FKBP51 silenced clones were grown till confluency in complete medium, proteins extracted and blotted for the expression of MR and FKBP51. Shown is a representative western blot of two silenced clones, 6a1 and 9b6. (D) Densitometric scan of MR and FKBP51 levels from western blots of 11 FKBP51 silenced clones grown as described in (C).
3.3.3. The effect of FKBP51 on aldosterone induced Isc.

To determine whether FKBP51 affects the aldosterone-induced transepithelial Na\(^{+}\) transport the aldosterone-induced increase in Isc was compared in cells expressing different levels of FKBP51. Fig. 14 depicts Isc values before and after aldosterone stimulation in FKBP51 over-expressing cells (Fig.14A) and in FKBP51 silenced cells (Fig.14B) in comparison to wild type cells. To exclude clone specific behavior three FKBP51 over-expressing clones and 2 FKBP51 silenced clones were assayed and data from 6-9 independent experiments was averaged.

The initial observation was that FKBP51 over-expressing clones have a ~26% lower Isc in complete medium. This effect is further augmented by the incubation in stripped serum and under these condition a ~48% inhibition of Isc by FKBP51 is seen (Fig.14A). However, both wild type and FKBP51 over-expressing cells responded to aldosterone. As shown in Fig.14C the over-expression of FKBP51 had a small and insignificant positive effect on aldosterone induced fractional increase in current (Isc(t)/ Isc(0)) .

Silencing FKBP51 in M1-MR cells strongly inhibited Isc by 47% in complete medium and had a mild inhibitory effect of 28% in stripped serum (Fig.14B). Analysis of aldosterone induced response demonstrated small but significant inhibition of aldosterone induced fractional change (Isc(t)/ Isc(0)) in these cells when compared to wild type (Fig.14D).

We conclude that FKBP51 does not inhibit aldosterone mediated Na\(^{+}\) transport but rather has a mild positive effect on aldosterone induced response. At the same time, both FKBP51 over-expressing and silencing cells had reduced Isc values compared to wild type cells in complete and in stripped serum medium. Since these results in over-expressed cells contradict results in silenced cells we cannot conclude about the effect of FKBP51 on basal Isc. Further interpretation of these results is discussed thoroughly under Discussion.

Independently, effects of FKBP51 on TER were evaluated. FKBP51 over-expression did not appear to have an effect on TER (Fig.14E). On the other hand, FKBP51 silencing caused a significant increase in TER which was maintained in the presence of amiloride.
Thus, it appears that FKBP51 loosens tight junctions of M1-MR cells and following it silencing the paracellular (amiloride-insensitive) resistance is higher.

**Fig. 14: The effect of FKBP51 on Isc in M1-MR cells.**

M1-MR cells and cell clones in which FKBP51 was overexpressed (M1-MR-FKBP51) or silenced (M1-MR-shRNA FKBP51) were cultivated on permeable supports in PC1 complete growth medium until the transepithelial resistance was >1 kohm*cm² and cells developed stable Isc values. Cells were then transferred to stripped serum containing medium and 24 hours later received 10nM aldosterone. Isc was determined in different times as above and the experiment was terminated by adding 10µM amiloride. (A) The figure summarizes Isc measurements from 9 independent experiments performed in duplicate or triplicate filters using three different M1-MR-FKBP51 clones. (B) Isc measurements of 6 independent experiments performed in duplicate or triplicate filters using two FKBP51 silenced clones in which the knockdown of FKBP51 was associated with only small decrease in MR. (C-D) Summary of aldosterone induced Isc fold change values (Isc(t)/Isc(0)) calculated from the Isc values of experiments in (A-B), respectively. (E-F) TER values in the experiments of (A-B), respectively. Bars are Means ±SEM. Probabilities were calculated using un-paired t-test * P<0.05, **P<0.005, *** p<0.0005, ****p<0.00005 vs. M1-MR cells.
The effect of FKBP51 on NF-κB activation.

One of the emerging roles of FKBP51 is its requirement for IKKα function and activation of NF-κB pathway (63, 91). It was also demonstrated that aldosterone induces NF-κB translocation into the nucleus and that such translocation inhibits Na⁺ transport in collecting duct cells. Therefore, we tested whether FKBP51 is involved in aldosterone mediated NF-κB activation in M1-MR cells. M1-MR and M1-MR-FKBP51 cells were grown in steroid depleted medium and then treated for 6 hours with aldosterone, 100 nM or 1 μM or vehicle. Activation of NF-κB pathway was determined by the level of p65 and p52 in both cytosolic and nuclear extracts. Proper separation of the two fractions has been verified by blotting for the nuclear protein Histone 2b. It was found that M1-MR cells grown in steroid depleted medium have activated nuclear p52 but inactive, exclusively cytosolic, p65. Aldosterone did not increase the level of nuclear p65 and p52 in these cells. In addition, FKBP51 overexpression in these cells did not affect distribution of either p65 or p52 (Fig. 15A).

To further assess possible involvement of NF-κB in the response to aldosterone we have determined translocation of the above proteins in distal colon. Accordingly, ADX rats were implanted with osmotic minipumps and effects of in vivo aldosterone treatment on nuclear translocation of p65 and p52 in distal colon were assessed. As shown in Fig. 15B the hormonal stimulation was not associated with translocation of neither p65 nor p52. The data seem however to indicate an increase in the total abundance of these proteins. We therefore conclude that the colonic response to aldosterone does not involve translocation of these proteins and, taken together with the negative result of Fig. 15A,
suggest that this is not the physiological role of FKBP51 in colonic epithelia and M1-MR cells.

**Fig. 15: Effect of FKBP51 on NF-κB activation.**

(A) M1-MR cells and two clones over-expressing FKBP51 (clone 1 and 2) were grown in PC1 medium and then in stripped serum containing medium for 2 days. Cells were incubated with the indicated concentrations of aldosterone for 6 hours. Cytosolic and nuclear protein was extracted and analyzed for the amounts of p65 and p52 using specific antibodies. Histone H2b has been used as a nuclear marker to determine fraction purity. (B) ADX rats were implanted with osmotic minipumps that delivered aldosterone for 5 or 24 hours. Colonic tissue was excised, cells homogenized and nuclei isolated as described under Methods. The abundances of p65 and p52 were compared in nuclei and total protein extract using Fibrillarin as nuclear marker. Each lane corresponds to a different rat.
3.3.5. The role of FKBP51 in oxidative stress induced cytotoxicity.

Recently it was reported that in 3T3-L1 fibroblasts FKBP51 resides also in mitochondria and translocates to the nucleus to protect cells against oxidative stress (92). Since the aldosterone-induced responses are known to be associated with oxidative stress we hypothesized that the up-regulation of FKBP51 in colon may provide protection against oxidative stress induced apoptosis. To test for this hypothesis wild type, FKBP51 over-expressing and silenced M1-MR cells were treated with 500 μM H$_2$O$_2$ for 5 hours and then cell viability was assayed by quantifying reduction of XTT. Silencing FKBP51 caused a 2-fold increase in peroxide mediated cytotoxicity in comparison with non-transfected cells. Over-expression of FKBP51 showed a tendency to increase cell viability and inhibited H$_2$O$_2$ toxicity in 3 out of 5 trails (Fig.16). This could be due to the fact that FKBP51 is expressed at sufficient amount in M1-MR cells thus increase of its level does not protect cells further against oxidative stress. This observation appears highly significant and raises the possibility that protection from oxidative stress is the reason for the colonic induction of FKBP51 by aldosterone. However, since this finding was made towards the very end of my work we were not able to further peruse it.

**Fig.16 : The effect of FKBP51 on H$_2$O$_2$ induced cytotoxicity.**

M1-MR, M1-MR-FKBP51 and M1-MR-shRNA FKBP51 cells were grown in complete medium in 96 well plate for 1 day and then incubated with or without 500 μM H$_2$O$_2$ for 5 hours. Cell viability was then determined by a 2 hours incubation with XTT. Viability of cells was measured as absorbance at 450 nM and peroxide mediated viability inhibition was calculated. Each line demonstrates a single experiment performed in quadruplicates. Red and blue colors relate to two different clones tested.
4. Discussion

In the current work we identified new aldosterone regulated genes by microarray analysis of distal colon, a native aldosterone responsive epithelial tissue. FKBP51 was identified as a gene whose mRNA is strongly induced by aldosterone. It should also be stressed that induction of FKBP51 by aldosterone reported here is far more profound than that of other aldosterone-induced genes reported in previous studies (29-33) indicating a significant function. Previous studies have demonstrated induction of FKBP51 by glucocorticoids in different tissues and recently regulation by aldosterone was shown in cardiomyocytes and endothelial cells (54-57,61,62). Yet, regulation by physiological concentrations of aldosterone has not been reported in epithelia and the involvement of this protein in the hormonal function has not been studied.


We demonstrate that induction of FKBP51 by aldosterone in distal colon of adrenalectomized rats is a true physiological effect since it is evoked by 10 nM aldosterone, a concentration that is well within the in vivo concentration of the hormone in rats under salt restriction (71). In addition, the effect is also seen in the presence of excess corticosterone and without adrenalectomy, which eliminates the possibility that its strong regulation by aldosterone is secondary to glucocorticoids elimination in ADX rats. Moreover, it has been demonstrated that in distal colon FKBP51 is induced by aldosterone but not by the natural glucocorticoid corticosterone. Only a small effect of corticosterone could be achieved by inhibition of 11β-hydroxysteroid dehydrogenase type 2 which is responsible for glucocorticoid conversion to inactive 11-dehydroxy metabolites in aldosterone responsive tissues. The relative insensitivity to CBX does not necessarily exclude enzymatic conversion as the reason for the difference between aldosterone and corticosterone. A second enzyme that can prevent mineralocorticoid response by glucocorticoids was described (93). In addition, other mechanisms for steroid specificity which do not require enzymatic modification have been reported (5, 94). In particular, it was suggested that binding of two different steroids to the same receptor may evoke different responses. i.e. it is possible that aldosterone binding to GR will induce FKBP51 while the binding of corticosterone to the same receptor will not. Irrespective of the above possibilities, since corticosterone failed to increase colonic FKBP51, this protein is expected to be regulated in colon under physiological conditions.
when both corticosterone and aldosterone are present in serum mainly by aldosterone. This however does not exclude the possibility that under stress conditions, when corticosterone is extremely elevated, FKBP51 can be regulated in the colon by glucocorticoid too.

The induction of FKBP51 in colon was apparent already after 2.5 hours but it was much stronger after 5 hours indicating that the induced protein is involved in a late response to the hormone. It is well established that aldosterone mediates its effects in two phases (25, 76). The early phase involves up-regulation of aldosterone-induced regulatory proteins leading to the activation of preexisting ENaC channels. The second, delayed phase, involves de novo synthesis of ENaC and Na⁺/K⁺ ATPase subunits as well as proteins involved in the negative-feedback regulation of aldosterone signaling. Taking into account the known inhibitory effect of FKBP51 on MR transcriptional activity, induction of FKBP51 in the late phase of the hormonal stimulation may suggest a role in negative feedback regulation of aldosterone response. However, such a mechanism was not supported by subsequent experiments in M1-MR cells.

One surprising observation was that most of the induction of FKBP51 was achieved by aldosterone concentrations that are much higher than those needed to fully saturate MR, but this effect was fully blocked by an MR antagonist and insensitive to the GR antagonist. A crucial role of MR is further demonstrated in M1 cells. Aldosterone augments FKBP51 in these cells only if MR is expressed. Inductions of protein only by high dose of aldosterone and yet full inhibition by spironolactone have been reported in at least two previous studies (95, 96) but so far no explanation was provided. Such behavior may be accounted by some permissive effect of MR on the response to GR. Several options for such permissive effect have been considered. The first is that induction of FKBP51 is evoked by a MR-GR hetero-dimer. Both MR and GR undergo dimerization upon hormone binding and such dimerization is an essential step in translocation to the nucleus and altering transcription. A number of studies have demonstrated also hetero-dimerization of MR and GR which adds to the versatility of the response (97-99). Induction of FKBP51 by an MR-GR hetero-dimer will be fully blocked by spironolactone and have a dose-response characteristic to the population of MR and GR. Such a mechanism has been recently proposed in BE(2)C and T84 cells (100). The ineffectiveness of RU486 to block the response to aldosterone may be accounted for by
its partial agonistic nature. Unlike other antagonists, RU486 does not prevent GR dimerization, translocation to the nucleus and binding to the hormone responsive element but inhibits only gene activation \((101, 102)\). No information exists whether a MR-GR dimer in which the MR site is occupied by aldosterone while the GR site is populated by RU486 is functional. An evidence for existence of such functional complex is provided in this work by the strong stimulatory effect of RU486 on FKBP51 induction by low aldosterone concentration. Additional evidence for existence of such functional complex was demonstrated in an early study in our laboratory using the toad bladder epithelium. In that study RU486 treatment augmented rather than inhibited the aldosterone-induced activation of ENaC \((103)\). Cooperative action between MR and GR was previously demonstrated in colon but physical interaction between the two proteins in this tissue was not shown \((97)\). In the current study the existence of MR-GR dimers in colon could be demonstrated by their co-immunoprecipitation from colonic extracts. This finding further supports the hypothesis of hetero-dimer mediated increase in FKBP51.

Additional possibilities for MR requirement of a GR mediated response are: (a) Necessity for the binding of both MR and GR to hormone responsive elements in the FKBP51 promoter. (b) The existence of a GR induced factor that acts as a transcriptional co-factor of MR mediated response or vice versa. However, none of these putative mechanisms accounts for the ineffectiveness of RU486 in inhibiting the induction of FKBP51.

Previous data showed that dexamethasone induces FKBP51 by direct binding of GR to GRE on FKBP51 gene in non-epithelial cells \((57)\). We expected that aldosterone too directly induces FKBP51. However, it turned out that the translation inhibitor CHX inhibits at least part of the response to 10nM aldosterone, suggesting the involvement of another aldosterone-induced protein. In addition, we showed that GR mediated FKBP51 induction by dexamethasone is a direct response while MR mediated FKBP51 induction by dexamethasone involves an intermediary protein like in case of MR mediated aldosterone response. We conclude that induction of FKBP51 by aldosterone is at least partially secondary to the synthesis of a mediator protein. However FKBP51 induction by glucocorticoids is a primary one.

Boyd et al \((104)\) reported that the up-regulation of ENaC mRNA by aldosterone depends also on protein synthesis. This study also demonstrated that Sgk-1, a classical mineralocorticoid induced protein kinase, is required for the induction of ENaC mRNA.
Zhang et al (88) further demonstrated that aldosterone-induced Sgk-1 relieves Dot1a-Af9 complex mediated transcriptional repression of α-ENaC. We therefore hypothesized that Sgk-1 may be the primary, CHX inhibitable, regulator of FKBP51. However, such an option was excluded by the lack of response to the SGK1 inhibitor GSK650394.

It should be noted that the partial inhibitory effect of CHX does not necessarily mean indirect induction of FKBP51 through another aldosterone induced protein. Additional possibility is a direct induction of FKBP51 by MR dependent transcription complex that involves protein with high turnover rate and thus a short lifetime. Illustration 2 summarizes the model suggested by the current results for the colonic induction of FKBP51 by aldosterone.

Illustration 2: A model of aldosterone mediated increase in FKBP51 mRNA.

Aldosterone induces FKBP51 by direct binding of activated MR-GR hetero-dimer to FKBP51 promoter. Partial inhibition of aldosterone mediated FKBP51 increase by cycloheximide suggests that induction of FKBP51 involves another aldosterone induced mediator protein. Alternatively, partial inhibitory effect of cycloheximide can be explained by direct FKBP51 increase by MR dependent transcription complex that involves protein with a high turnover rate.

Since kidney is an important organ in the maintenance of Na⁺ and K⁺ transport and thus in blood volume regulation we studied regulation of FKBP51 in this tissue as well. Our initial in-vivo studies using saturating aldosterone concentration showed a marked increase in FKBP51 RNA in kidney but the subsequent ex-vivo studies using defined physiological concentration of 10 nM showed no induction of FKBP51 in kidney collecting duct. Since the induction of this gene was restricted in kidney to high doses of aldosterone its importance in aldosterone response is questionable. The lack of strong
increase in FKBP51 in kidney is probably due to higher constitutive expression level of this protein in kidney compared to colon. A similar diverse regulation in kidney and colon was observed for additional genes such as CHIF, β₂γ ENaC and k-Ras2 (20, 29, 44, 45, 74). It has been proposed that these differences represent somewhat different aldosterone-induced mechanisms in rapidly (colon) vs. slow (CCD) proliferating cells.

4.2. The possible roles of FKBP51 in aldosterone response.

The next part of my thesis explored the potential roles of FKBP51 in the colonic response to aldosterone. In principle, two classes of mechanisms were considered. The first is response mediated by the well established interaction of FKBP51 with either MR or GR and inhibiting their transcriptional activity. The second is a receptor independent mechanism such as the previously demonstrated activation of NF-κB or the recent finding of protection from oxidative stress (63, 91, 92).

According to the accepted model cytoplasmic, hormone free, GR or MR associates with a superchaperone complex that consists of HSP90 dimer, p23 and FKBP51. FKBP51 inhibits transcriptional activity of GR and MR. Binding of corticosteroids to the steroid receptors transfers the equilibrium of the superchaperone complexes towards the FKBP52-containing ones which facilitates dimerization and nuclear transport of GR and MR (47-52). FKBP51 was shown to be induced by glucocorticoids in several tissues and cell lines and considered to play a role in negative feedback inhibition (54-57). We therefore hypothesized that the strong up-regulation of FKBP51 by aldosterone identified in this study may participate in negative feedback regulation of aldosterone signaling. Such negative feedback may serve one of two purposes: (1) synchronize responses in cells expressing different levels of MR or GR i.e. the higher is the receptor level more FKBP51 will be induced and the physiological response will be suppressed to the same level achieved in cell expressing less receptors. (2) A “built in” shut-off mechanism that will terminate the effect after chronic exposure (that induces FKBP51) before aldosterone is removed from the bloodstream.

To address a possible role of FKBP51 in the aldosterone-induced augmentation of Na⁺ transport we have established M1-CCD derived cell lines that respond to aldosterone and in which FKBP51 is either over-expressed or silenced. Response of these cells to
aldosterone did not support the above possibilities but raises other interesting possibilities. The first interesting observation was that FKBP51 affects the abundance of the MR protein in M1-MR cells. Marked increase in MR abundance was observed in two out of three over-expressing clones and reduction of MR was observed in 9 out of 11 silenced clones. For the study of FKBP51 role in Na$^+$ transport we used all three FKBP51 over-expressing clones, and the two silenced FKBP51 clones in which only a small reduction of MR was observed.

The initial inspection made was that both silencing and over-expression of FKBP51 appears to lower steady state Isc in complete medium and this effect is maintained after a 24 incubation in steroid free medium. This incubation largely decreased Isc irrespective of FKBP51 level. Under stripped serum conditions the inhibitory effect of FKBP51 over-expression on Isc was stronger than the inhibitory effect of FKBP51 silencing. Since our commercial complete medium included different growth factors and steroids it is currently not known removal which of these components caused a stronger decrease in Isc in over-expressed cells. Irrespective, because the Isc was inhibited in both over-expressed and silenced cells, we cannot attribute these effects to FKBP51.

Since, Isc values prior the addition of aldosterone are different, comparing the response to the hormone requires normalization to the initial Isc. This can be done in two ways: (1) Calculating the incremental increase in Na$^+$ flux i.e. Isc after aldosterone minus Isc before aldosterone. (2) Calculating the fractional change i.e. Isc after aldosterone/Isc before aldosterone. The correct way to analyze data is mechanism dependent. The first approach assumes that the aldosterone induced increase in Isc is independent of the basal Isc. This might be the case for an aldosterone-induced de novo channel synthesis. The second approach however assumes that the response to aldosterone is a function of the basal Isc. Such behavior is predicted when the hormone affects pre-existing equilibrium between closed and open channels or membranal and intracellular channels. It was previously demonstrated that in M1 cells SGK1, a kinase that regulates Nedd4 dependent internalization of ENaC, is required for aldosterone mediated response (105). In addition, it is well known that de novo channel synthesis lags behind the aldosterone induced increase in Isc (25). Since most of the Isc response to aldosterone in our system takes places after 6 hr, comparing cells by looking at the fractional aldosterone-induced increase in Isc seems the appropriate way.
Analysis of aldosterone response did not support the assumption of FKBP51 involvement in negative feedback inhibition of aldosterone response. On the contrary, silencing FKBP51 had a mild but significant inhibitory effect, while over-expression of FKBP51 had a small insignificant stimulatory effect. This behavior was verified by averaging data from several clones and large number of independent experiments. We conclude that since FKBP51 has no or only a small positive effect on aldosterone response, the up-regulation of FKBP51 by a large factor in colon is not an essential step in aldosterone mediated Isc increase. In addition, the small effect of FKBP51 on aldosterone response can be attributed to the positive effect this protein has on MR level in M1 cells.

As described above, it was found that FKBP51 significantly affects the abundance of the MR protein. It is well known that FKBP51 functions to inhibit transcriptional activity of corticosteroid receptors, but its role in maturation of active corticosteroid receptors is not clear. Only recently, it was demonstrated in prostate cancer cells that FKBP51 over-expression promotes assembly of HSP90 chaperone complex and facilitates association of this complex with AR thus increasing the number of AR molecules that undergo androgen binding (106). Our result suggests that FKBP51 may stimulate maturation of MR complexes as well. This may suggest another role for induction of FKBP51. We hypothesized that maximal response to the hormone requires not only sufficiently high levels of circulating aldosterone but also up-regulation of MR. In that case FKBP51 may be needed to promote maturation of newly synthesized MR. However, we did not detect aldosterone mediated increase in MR protein in colon. Alternative possibility is the requirement of FKBP51 in maturation of newly synthesized MR as a result of a higher ligand dependent turnover rate of MR complexes. It was previously shown that proteosome activity is necessary for optimal MR transactivation (107). This finding is consistent with other evidences for continuous proteosome-mediated turnover of highly active transcription factors on their cognate promoters (108-111). Since we did not inspect reduction of MR level in colon following aldosterone stimulation it is possible that MR level is maintained during aldosterone response by newly synthesized, FKBP51 incorporating, MR complexes. Further studies are needed to explore this issue.

Another mechanism considered for the role of FKBP51 in the aldosterone response is activation of the NF-κB pathway. Bouwmeester et al. (63) demonstrated that FKBP51 interacts with IKKα and plays an essential role in the overall signaling process triggered
by NF-κB activation. Other studies suggested that activation of NF-κB has either negative or positive effects on Na⁺ transport (64, 65). However, we were unable to demonstrate effects of aldosterone on NF-κB translocation in either distal colon or M1 cells. We also did not see effect of FKBP51 over-expression on NF-κB nuclear translocation.

Finally, we explored the possibility that the aim of FKBP51 is to protect cells from an oxidative stress that may accompany the response to aldosterone. Gallo et al (92) has recently reported that FKBP51 protects cells against oxidative stress induced apoptosis. In addition they demonstrated that this protein resides also in the mitochondria and is translocated to the nucleus in response to oxidative stress induced by the application of H₂O₂. To further assess possible FKBP51 role in oxidative stress, we measured sensitivity to H₂O₂ of FKBP51 silenced and overexpressing M1 cells. Indeed the silencing of FKBP51 led to a 2 fold increase in the sensitivity of the cells to H₂O₂. The overexpression of FKBP51 on the other hand appeared to be without effect and did not provide protecting effect in addition to that seen in FKBP51 expressing normal cells. It is well known that the maximal response of aldosterone is limited by the ATP supply to the Na⁺/K⁺ ATPase and a major group of aldosterone induced proteins are mitochondrial enzymes involved in oxidative phosphorylation (25). Also, spironolactone is commonly used as therapeutics in heart failure antagonizing an aldosterone induced oxidative stress. Thus, protecting cells from oxidative stress is a likely possibility for the role of FKBP51.

In summary, the current study identifies FKBP51 as a major aldosterone induced gene in distal colon, induced following long-term exposure to the hormone. Dose-response relationships, antagonists sensitivity and other findings suggest that maximal induction of FKBP51 requires occupancy of both MR and GR with an obligatory role of MR. These data are best explained by assuming that the response is mediated by MR-GR dimer. Several putative roles for the effect of FKBP51 in the aldosterone induced cascade have been explored. Evidence were provided for two such mechanisms: (a) Augmenting MR level and (b) protecting cells from oxidative stress likely to accompany the elevated rate of Na⁺ transport.
5. References


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鬘תר המחקר היה וודא עם נוכחות הגלובלים על ידי אלדוסטרון. FKBP51, החלבון הקיים של קומפלקס של קרום סטרואידים (Hsp90), נמצאות בין מבוקרים על ידי כיוון פיזיולוגים של אלדוסטרון בăngברת להטיה וריכוז תאים. נמצאות גם בكورونا של קולטנים סטרואידים וברקゴールדון (IKKα). זה נמצא עובר הגברה חזקה בתגובה לשפעון ההורמונלי. הגברה זו אומתה גם ברמת החלבון. מדידות ברקמה מבודדת אפינו את קצב הגברה ויחסי ריכוז תגובת FKBP51 mRNA, ומצא כי FKBP51 מגבר על ידי ריכוזים פיזיולוגים של אלדוסטרון בתגובה ל侮辱 (תור מ-2.5 שעה). עלילה ברמה של FKBP51 mRNA ומצטברת партиות (MR) הʏורת ווחי הורמון והורמון המבוקרים לפני הReusable למול של חלבון ממילולייקורטיקואידיים. הפרטים, המבחנים של לקולמוס, אף על פי שפגש fullname MR, כדי לנהל את הקולטנים, MR-486 ואל-גרו רוועד עם זיהוי migrated MR-486. בלוקה גם על ידי מעובבים של החלבונים, פונקציה של כיוון פיזיולוגים של אלדוסטרון הדליות להטיה וה껸וץ. MR שולCUS הידיעה עם הורמון המבוקרים לפני הReusable למול של חלבון ממילולייקורטיקואידיים. פריטים המבחנים של לקולמוס, אף על פי שפגש fullname MR-486 ואל-גרו רוועד עם זיהוי migrated MR-486. בלוקה גם על ידי מעובבים של החלבונים, פונקציה של כיוון פיזיולוגים של אלדוסטרון הדליות להטיה וה 生命周期י ANP עם הורמון המבוקרים לפני הReusable למול של חלבון ממילולייקורטיקואידיים. פריטים המבחנים של לקולמוס, אף על פי שפגש fullname MR-486 ואל-גרו רוועד עם זיהוי migrated MR-486. בלוקה גם על ידי מעובבים של החלבונים, פונקציה של כיוון פיזיולוגים של אלדוסטרון הדליות להטיה וה 生命周期י ANP עם הורמון המבוקרים לפני הReusable למול של חלבון ממילולייקורטיקואידיים. פריטים המבחנים של לקולמוס, אף על פי שפגש fullname MR-486 ואל-גרו רוועד עם זיהוי migrated MR-486. בלוקה גם על ידי מעובבים של החלבונים, פונקציה של כיוון פיזיולוגים של אלדוסטרון הדליות להטיה וה 生命周期י ANP עם הורמון המבוקרים לפני הReusable למול של חלבון ממילולייקורטיקואידיים. פריטים המבחנים של לקולמוס, אף על פי שפגש fullname MR-486 ואל-גרו רוועד עם זיהוי migrated MR-486. בלוקה גם על ידי מעובבים של החלבונים, פונקציה של כיוון פיזיולוגים של אלדוסטרון הדליות להטיה וה 生命周期י ANP עם הורמון המבוקרים לפני הReusable למול של חלבון ממילולייקורטיקואידיים. פריטים המבחנים של לקולמוס, אף על פי שפגש fullname MR-486 ואל-גרו רוועד עם זיהוי migrated MR-486. בלוקה גם על ידי מעובבים של החלבונים, פונקציה של כיוון פיזיולוגים של אלדוסטרון הדליות להטיה וה 生命周期י ANP עם הורמון המבוקרים לפני Hsp90, מבפק 보면 ת tôח וה fopenפאלים שניים על ידי אלדוסטרון. המধמים הן וירטואליות או執行י מלאי מלחמות החממות. כש suffice MR-486aksi בין הורמון המבוקרים לפני Hsp90, מבפק 보면 ת tôח וה fopenפאלים שניים על ידי אלדוסטרון. המধמים הן וירטואליות או�行י מלאי מלחמות החממות. כש suffice MR-486aksi בין הורמון המבוקרים לפני Hsp90, מבפק 보면 ת tôח וה fopenפאלים שניים על ידי אלדוסטרון. המধמים הן וירטואליות או�行י מלאי מלחמות החממות. כש suffice MR-486aksi בין הורמון המבוקרים לפני Hsp90, מבפק 보면 ת tôח וה fopenפאלים שניים על ידי אלדוסטרון. המধמים הן וירטואליות או�行י מלאי מלחמות החממות. כש suffice MR-486aksi בין הורמון המבוקרים לפני Hsp90, מבפק 보면 ת tôח וה fopenפאלים שניים על ידי אלדוסטרון. המধמים הן וירטואליות או�行י מלאי מלחמות החממות. כש suffice MR-486aksi בין הורמון המבוקרים לפני Hsp90, מבפק 보면 ת tôח וה fopenפאלים שניים על ידי אלדוסטרון. המधמים הן וירטואליות או�行י מלאי מלחמות החממות. כש suffice MR-486aksi בין הורמון המבוקרים לפני Hsp90, מבפק 보면 ת tôח וה fopenפאלים שניים על ידי אלדוסטרון. המধמים הן וירטואליות או�行י מלאי מלחמות החממות. כש suffice MR-486aksi בין הורמון המבוקרים לפני Hsp90, מבפק 보면 ת tôח וה fopenפאלים שניים על ידי אלדוסטרון. המধמים הן וירטואליות או�行י מלאי מלחמות החממות. כש suffice MR-486aksi בין הורמון המבוקרים לפני Hsp90, מבפק 보면 ת tôח וה fopenפאלים שניים על ידי אלדוסטרון. המধמים הן וירטואליות או�行י מלאי מלחמות החממות. כש suffice MR-486aksi בין הורמון המבוקרים לפני Hsp90, מבפק 보면 ת tôח וה fopenפאלים שניים על ידי אלדוסטרון. המধמים הן וירטואליות או�行י מלאי מלחמות החממות. כש suffice MR-486aksi בין הורמון המבוקרים לפני Hsp90, מבפק 보면 ת tôח וה fopenפאלים שניים על ידי אלדוסטרון. המধמים הן וירטואליות או�行י מלאי מלחמות החממות. כש suffice MR-486aksi בין הורמון המבוקרים לפני Hsp90, מבפק 보면 ת tôח וה fopenפאלים שניים על ידי אלדוסטרון. המধמים הן וירטואליות או�行י מלאי מלחמות החממות. כש suffice MR-486aksi בין הורמון המבוקרים לפני Hsp90, מבפק 보면 ת tôח וה fopenפאלים שניים על ידי אלדוסטרון. המধמים הן וירטואליות או�行י מלאי מלחמות החממות. כש suffice MR-486aksi בין הורמון המבוקרים לפני Hsp90, מבפק 보면 ת tôח וה fopenפאלים שניים על ידי אלדוסטרון. המধמים הן וירטואליות או�行י מלאי מלחמות החממות. כש suffice MR-486aksi בין הורמון המבוקרים לפני Hsp90, מבפק 보면 ת tôח וה fopenפאלים שניים על ידי אלדוסטרון. המধמים הן וירטואליות או�行י מלאי מלחמות החממות. כש suffice MR-486aksi בין הורמון המבוקרים לפני Hsp90, מבפק 보면 ת tôח וה fopenפאלים שניים על ידי אלדוסטרון. המধמים הן וירטואליות או�行י מלאי מלחמות החממות. כש suffice MR-486aksi בין הורמון המבוקרים לפני Hsp90, מבפק 보면 ת tôח וה fopenפאלים שניים על ידי אלדוסטרון. המধמים הן וירטואליות או�行י מלאי מלחמות החממות. כש suffice MR-486aksi בין הורמון המבוקרים לפני Hsp90, מבפק 보면 ת tôח וה fopenפאלים שניים על ידי אלדוסטרון. המধמים הן וירטואליות או�行י מלאי מלחמות החממות. כש suffice MR-486aksi בין הורמון המבוקרים לפני Hsp90, מבפק 보면 ת tôח וה fopenפאלים שניים על ידי אלדוסטרון. המধמים הן וירטואליות או�行י מלאי מלחמות החממות. כש suffice MR-486aksi בין הורמון המבוקרים לפני Hsp90, מבפק 보면 ת tôח וה fopenפאלים שניים על ידי אלדוסטרון. המধמים הן וירטואליות או�行י מלאי מלחמות החממות. כש suffice MR-486aksi בין הורמון המבוקרים לפני Hsp90, מבפק 보면 ת tôח וה fopenפאלים שניים על ידי אלדוסטרה Shortly after the hormone effect, FKBP51 mRNA was found to increase greatly in response to the hormone. The increase was also observed in the level of the protein. Measurements in isolated muscle confirmed the rate and relative concentration of the response. It was found that FKBP51 was increased by physiological concentrations of aldosterone beyond a certain point. The increase in FKBP51 mRNA was at least partially due to inhibition of protein synthesis, indicating a dual effect on the expression of other genes also affected by the hormone. In addition, at lower concentrations of aldosterone RU486 led to an increase in FKBP51. The data thus suggest a possible role of FKBP51 in the assembly and stabilization of MR during the response to aldosterone. Further studies involving the use of nongenetic models confirmed the presence of Na⁺ in M1-MR. Therefore, another possible role of FKBP51 is to enhance the protection of the cells from the acid stress accompanying the increase in Na⁺ and ATP consumption during the response to aldosterone.