(n = 24) and 1.8 ± 2.3 (n = 26). n, number of recordings from different chemosensilla. Trehalose response difference was statistically significant by t-test ($P < 0.001$).
25. Transgenic flies carrying *Tre1* cDNA under the heat-shock promoter were generated by P element–mediated germ line transformation. A 1.4-kb Eco RI–Xho I fragment containing the entire *Tre1* cDNA was cloned into the Eco RI site in the P-element transformation vector, pKB255 [K. Basler, unpublished data; K. Basler, K. Yen, A. Tomlinson, E. Hafen, *Genes Dev.* **4**, 728 (1990)], placing it under the control of the *Drosophila* hsp70 promoter. This construct was then used to generate germ line transformants by injecting into w^{1118}; Dr^1/TMS, Sb^1 P[ry^+, Δ2-3]99B embryos. Transformants were selected, and homozygous lines were established.
26. In situ hybridization was done as described [T. R. Bhatt, P. A. Taylor III, F. M. Horodyski, *Biotechniques* **23**, 1000 (1997)] with minor modifications. The DIG-labeled antisense and sense riboprobes were produced by in vitro transcription by T7 and Sp6 RNA polymerase with a 244-bp *Tre1* cDNA fragment as a template, respectively. Antidigoxygenine antibody conjugated to peroxidase (Pierce) was applied at a dilution of 1:500 overnight at 4°C, and signals were enhanced with biotin-tyramide (NEN). After tyramide signal amplification reaction, streptavidin-TC (Caltag) was applied at a dilution of 1:100. Sensory neurons were stained with a neuron-specific monoclonal antibody 22C10 using anti-mouse IgG antibody conjugated to Cy5 (Chemicon International Inc.) diluted to 1/100 as the secondary antibody. Nuclei were counter stained with propidium iodide. The triple-stained images were visualized with a Zeiss LSM410 confocal laser scan microscope equipped with a Kr/Ar and a He/Ne laser.
27. We have sequenced *Tre1* cDNA clones obtained from trehalose high-sensitive and low-sensitive strains and found a few possible mutation sites, but further study is needed to verify that the mutation is directly involved in the trehalose sensitivity difference.
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32. We are grateful to K.-I. Kimura for *poxn* and K. Basler for plasmid. EP lines and other fly strains were provided by the Bloomington stock center and the Berkeley *Drosophila* Genome Project. The monoclonal antibody 22C10 was obtained from the Developmental Studies Hybridoma Bank at The University of Iowa. We thank T. Hirozane for her initial extensive attempt on this project and T. Hirozane and T. Tamura for their help in sequencing. We also thank Y. Mitsuiki, M. Haruta, M. Yamasaki for their technical assistance and C. Montell for critically reading the early version of our manuscript. Supported by grants from the Ministry of Education, Science, Sports and Culture of Japan and by a Human Frontier Science Program grant to T.T. The *Tre1* cDNA sequences have been deposited in DDBJ (accession number AB034204). This paper is dedicated to the memory of the late Dr. Toshihide Kikuchi.

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Activating Mineralocorticoid Receptor Mutation in Hypertension Exacerbated by Pregnancy

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Hypertension and pregnancy-related hypertension are major public health problems of largely unknown causes. We describe a mutation in the mineralocorticoid receptor (MR), S810L, that causes early-onset hypertension that is markedly exacerbated in pregnancy. This mutation results in constitutive MR activity and alters receptor specificity, with progesterone and other steroids lacking 21-hydroxyl groups, normally MR antagonists, becoming potent agonists. Structural and biochemical studies indicate that the mutation results in the gain of a van der Waals interaction between helix 5 and helix 3 that substitutes for interaction of the steroid 21-hydroxyl group with helix 3 in the wild-type receptor. This helix 5–helix 3 interaction is highly conserved among diverse nuclear hormone receptors, suggesting its general role in receptor activation.

Although blood pressure is normally reduced throughout gestation, about 6% of pregnancies are complicated by the development of hypertension, raising the risk of pre-eclampsia, a hypertensive disorder of pregnancy that increases maternal and perinatal mortality (*1*). The factors responsible for these developments are unknown; however, the prompt resolution of many cases with delivery suggests pregnancy-specific factors.

Mutations that change renal salt reabsorption alter blood pressure (*2*). For example, heterozygous loss-of-function mutations in the mineralocorticoid receptor (MR; locus symbol *NR3C2*), a member of the nuclear receptor family, cause pseudohypoaldosteronism type 1 (PHA1), a disease featuring salt wasting and hypotension (*3*). Normally, activation of MR by the steroid hormone aldosterone raises renal salt reabsorption by increasing activity of the epithelial sodium channel of the distal nephron.

To determine if gain-of-function mutations in *MR* could cause increased renal salt reabsorption and hypertension, we screened *MR* in 75 patients with early onset of severe hypertension (*4*). A 15-year-old boy with severe hypertension, suppressed plasma renin activity, low serum aldosterone, and no other underlying cause of hypertension was heterozygous for a missense mutation, resulting in substitution of leucine for serine at codon 810 (Fig. 1A). The S810L mutation lies in the MR hormone-binding domain (HBD), altering an amino acid that is conserved in all MRs from *Xenopus* to human (*5*) but not found in other nuclear receptors (Fig. 1B). S810L was not detected in 160 control chromosomes.

The impact of MR bearing S810L (MR_{L810}) was assessed by clinical and biochemical studies. Twenty-three relatives of the proband were evaluated. Remarkably, 11 had been diagnosed with severe hypertension before age 20, a rare trait in the general population (*6*), whereas the remainder had unremarkable blood pressures (Fig. 1C). These findings suggest Mendelian segregation of hypertension. MR_{L810} precisely cosegregated with early-onset hypertension in this family (Fig. 1C), providing strong evidence of linkage. The maximum lod score (logarithm of the odds ratio for linkage) was 5.24 at a recombination fraction of zero (odds of 174,000:1 in favor of linkage) (*7*).

Comparison of the clinical features of MR_{L810} carriers and noncarriers revealed a marked increase of blood pressure among carriers even though they were taking antihypertensive medication, as well as suppression of aldosterone secretion (Table 1). There was a nonsignificant trend toward lower serum potassium among carriers, and there were no significant effects of gender or age on phenotypic expression of MR_{L810}. Of note, three deceased pedigree members with early-onset hypertension all died of heart failure before age 50.

To investigate the functional effects of the

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S810L mutation, we expressed wild-type MR (MR_{WT}) or MR_{L810} in Cos-7 (African green monkey kidney) cells and measured induction of luciferase under control of the MR-sensitive mouse mammary tumor virus (MMTV) promoter (*8*). The activities of MR_{WT} and MR_{L810} in response to aldosterone were indistinguishable (Fig. 2A). However, in the absence of added steroid, MR_{L810} showed 27% of maximal activity ($P < 0.001$ versus MR_{WT}), indicating constitutive activity of the apo-receptor or activation by endogenous compounds (Fig. 2A). Constitutive MR_{L810} activity was also seen in HeLa cells. This constitutive activity may account for or contribute to the hypertension seen in patients harboring MR_{L810}.

We next tested MR activation by a variety of steroids. Steroids bearing 21-hydroxyl groups, such as aldosterone, bind and activate MR_{WT} (*9*) and similarly activate MR_{L810} (Fig. 2B). Steroids with 17-keto groups, including estradiol and testosterone, activate neither MR_{WT} nor MR_{L810}. Twenty-one–carbon steroids with neither modification, such as progesterone, normally act as antagonists, binding but not activating MR_{WT} (*9*). In contrast to their action on MR_{WT}, these steroids were all potent activators of MR_{L810} (Fig. 2C). For example, the dose-response curve for activation of MR_{L810} by progesterone is indistinguishable from that seen with MR_{WT} and aldosterone (Fig. 2, A and D). Thus, activation of MRs with the S810L mutation no longer requires a steroid 21-hydroxyl group. Similarly, another MR antagonist, the clinically used drug spironolactone, is also a potent agonist of MR_{L810} (Fig. 2E); we infer that this medication is contraindicated in MR_{L810} carriers.

MR activates transcription as a dimer (*10*). Because MR_{L810} is heterozygous in affected individuals, we tested progesterone-mediated MR activation in the presence of varying ratios of MR_{WT} and MR_{L810} (Fig. 2F). Activation was intermediate between that expected for completely active and completely inactive heterodimers, consistent with partial activation of heterodimers.

Progesterone levels normally increase 100-fold in pregnancy, reaching concentrations of 500 nM (*11*); this suggests that females with MR_{L810} might develop severe hypertension in pregnancy. Two MR_{L810} carriers have undergone five pregnancies; all have been complicated by marked exacerbation of hypertension. For example, blood pressure decreased early in the first pregnancy of one carrier, but then rose dramatically, reaching 170/130 mm Hg at 28 weeks despite antihypertensive therapy. This was accompanied by development of low serum potassium with marked renal potassium wasting. Aldosterone levels, which normally increase 10-fold in pregnancy, were undetectable (*12*). Of note, there were no proteinuria, edema, or neurologic changes, excluding pre-eclampsia. Because of still worsening blood pressure (210/120 mmHg), at 34 weeks a Caesarian section was performed, and the patient gave birth to a healthy son. Two subsequent pregnancies followed similar courses, and the patient was advised to avoid further pregnancy. The other carrier's two pregnancies were both complicated by severe exacerbation of hypertension, precipitating delivery in the sixth and seventh months of gestation, also with advice to avoid further pregnancy. These findings strongly support progesterone's in vivo agonism of MR_{L810}.

The structures of the HBDs of steroid receptors are highly similar (*13*). The MR and progesterone receptor (PR) differ by only 3 of 18 residues lining the hormone-binding cavity (*13*, *14*), permitting modeling of the MR HBD on the basis of the known structure of PR (*15*) in order to generate testable hypotheses about the mechanism underlying the S810L mutation. In the resulting MR_{L810} model, the L810 side chain in helix 5 projects into the ligand-binding cavity, potentially forming van der Waals interactions with both A773 of helix 3 and the C19 methyl group of the steroid (Fig. 3A). In contrast, in the model of MR_{WT}, S810 interacts with only the C19 methyl group (Fig. 3B). Of note, the steroid 21-hydroxyl group required for activation of MR_{WT} interacts with N770 in helix 3 (*14*).

We tested the importance of these inter-

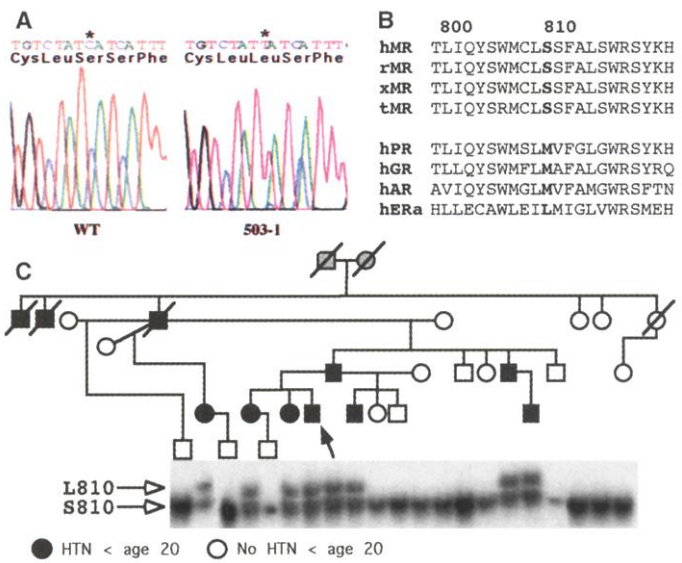

Fig. 1. MR_{L810} cosegregates with early hypertension in kindred AMR 503. (**A**) Sequence of a novel MR variant identified in K503. The DNA sequence corresponding to the sense strand of codons 808 to 812 of the wild-type (left) and mutant MR (right) are shown (*4*). The asterisks indicate the position of a C→T substitution, which changes codon 810 in the HBD from serine to leucine. (**B**) Sequence alignment of steroid receptors. Corresponding segments of the HBDs of the mineralocorticoid (MR), progesterone (PR), glucocorticoid (GR), androgen (AR), and estrogen α (ERα) receptors are shown. MR sequences are from *Homo sapiens* (h), the rat *Rattus norvegicus* (r), the toad *Xenopus laevis* (x), and the tree shrew *Tupaia belangeri* (t) (*5*). S810 is conserved among all known MRs, yet is not seen in other members of the steroid hormone receptor family (*5*). (**C**) Cosegregation of early hypertension and MR_{L810} in kindred 503. Family members diagnosed with hypertension before the age of 20 are shown as filled symbols and those without this trait are shown as unfilled symbols. Individuals of unknown phenotypic status are shown as shaded symbols. The index case is indicated by the black arrow. The SSCP genotype of exon 6 of MR (*4*) is shown below the symbol corresponding to the appropriate kindred members; the positions of the S810 and L810 variants are indicated by open arrows.

Table 1. Clinical features of MR_{L810} carriers (+) and noncarriers (−) in kindred 503. All values are the mean ± SEM. Statistical comparisons were assessed by the Mann-Whitney U test. Normal values: SBP < 140 mmHg; DBP < 90 mmHg; serum K^+: 3.8 to 5.2 mM; serum HCO_3^-: 23 to 30 mM; serum aldosterone 4 to 15 (ng/dl); and urinary aldosterone: 6 to 31 μg/24 hours. Urinary aldosterone was measured in three MR_{L810}^+ and four MR_{L810}^- individuals. Abbreviations: HTN, hypertension; SBP, systolic blood pressure; DBP, diastolic blood pressure; HCO_3^-, bicarbonate.

Clinical parameter	MR_{L810}^+ (n = 8)	MR_{L810}^- (n = 11)	P
Age	29.1 ± 6.3	32.9 ± 8.1	0.88
HTN < age 20	100%	0%	<0.0001
Anti-HTN medication	1.5 ± 0.27	0.2 ± 0.12	0.001
SBP (mmHg)	167 ± 11	126 ± 10	0.014
DBP (mmHg)	110 ± 6	78 ± 6	0.002
Serum K^+ (mM)	3.91 ± 0.18	4.36 ± 0.11	0.08
Serum HCO_3^- (mM)	27.1 ± 0.87	26.4 ± 0.83	0.59
Serum aldosterone (ng/dl)	2.48 ± 0.68	12.1 ± 2.96	0.008
Urinary aldosterone (μg/24 hours)	<2	7.75 ± 1.55	0.03

actions by modifying both the steroid and the receptor. The progesterone derivative 19-norprogesterone (19-NP), lacking the C19 methyl group, fully activated MR_{L810}, indicating that the L810–C19 methyl interaction is not required for MR_{L810} activation (Fig. 2C). Substitution of methionine, with a longer side chain, at position 810 retained activation by 19-NP, whereas substitution of amino acids with shorter side chains such as valine or alanine led to progressive loss of receptor activation by 19-NP, but not aldosterone (Fig. 3C). That this effect involves interaction with A773 in helix 3 is supported by substitution at this position. MR with a V810-G773 pair showed no activation by 19-NP, but activation was restored in the V810-A773 pair (Fig. 3C). This second-site complementation supports the importance of helix 3–helix 5 interaction in the activation of MR_{L810} by steroids lacking 21-hydroxyl groups.

These observations suggest that progesterone binding to MR_{L810} results in an additional van der Waals interaction; this should increase receptor affinity for progesterone. MR_{WT} and MR_{L810} show indistinguishable dissociation constants for aldosterone (K_d = 1.10 ± 0.09 nM versus 1.06 ± 0.12 nM, respectively) (Fig. 4A) (*16*). In contrast, progesterone, 19-NP, and spironolactone all show increased competition with aldosterone for binding to MR_{L810}, indicating substantially higher affinity for the mutant receptor (Fig. 4, B to D). For example, the concentration of spironolactone required to compete 50% of aldosterone binding was 10-fold lower for MR_{L810} than for MR_{WT} (Fig. 4D).

The finding of a helix 5–helix 3 interaction critical for MR_{L810} activation by progesterone led us to examine other nuclear steroid receptors for similar interactions. Intriguingly, the residues corresponding to MR amino acids 810 and 773 that are found in PR (M759 and G722) (*13*), the estrogen receptor (L387 and A350) (*17*), and even the nonsteroid retinoid X receptor (RXR) (L314 and A277) (*18*) all show van der Waals interactions in their respective crystal structures. Indeed, all steroid hormone receptors and RXRs have either methionine-glycine or leucine-alanine pairs at the corresponding helix 5 and helix 3 positions, respectively (*5*, *12*). Only MR_{WT} violates this rule, and the S810L mutation restores this interaction (Fig. 3B). The conservation of this interaction and its role in MR_{L810} activation by progesterone suggest its general significance in nuclear receptor activation.

Together, these findings define a new Mendelian form of hypertension featuring marked exacerbation in pregnancy. The mutation demonstrates the ability of a single amino acid substitution to dramatically alter nuclear receptor ligand selectivity and activation. It will be of interest to determine the effects of the S810L mutation on the subcellular distribution of MR and on binding of coactivators and corepressors.

Normal pregnancy is characterized by protean physiological changes. Efforts to identify novel endocrine factors in pregnancy-related hypertension and pre-eclampsia have been unsuccessful (*1*). The demonstration that one form of pregnancy-related hypertension is attributable to aberrant action of a normal pregnancy-related hormone raises the question of whether similar mechanisms might underlie other forms. It is noteworthy that a substantial fraction of women with pregnancy-related hypertension and pre-eclampsia show suppression of the renin-aldosterone axis like that seen in this kindred.

The mechanism of early-onset hypertension in men in this kindred is unclear, because levels of free progesterone in males are normally low (*14*). This observation suggests either that the observed constitutive receptor activation is sufficient to cause hypertension or that another steroid acts as an MR_{L810} agonist. Interestingly, free levels of 17-hydroxyprogesterone (17-HP), an agonist of MR_{L810} (Fig. 2C), rival those of aldosterone in men (*11*), suggesting that gain of 17-HP agonist activity may contribute to hypertension in men with MR_{L810}.

Activation of MR_{WT} requires a steroid

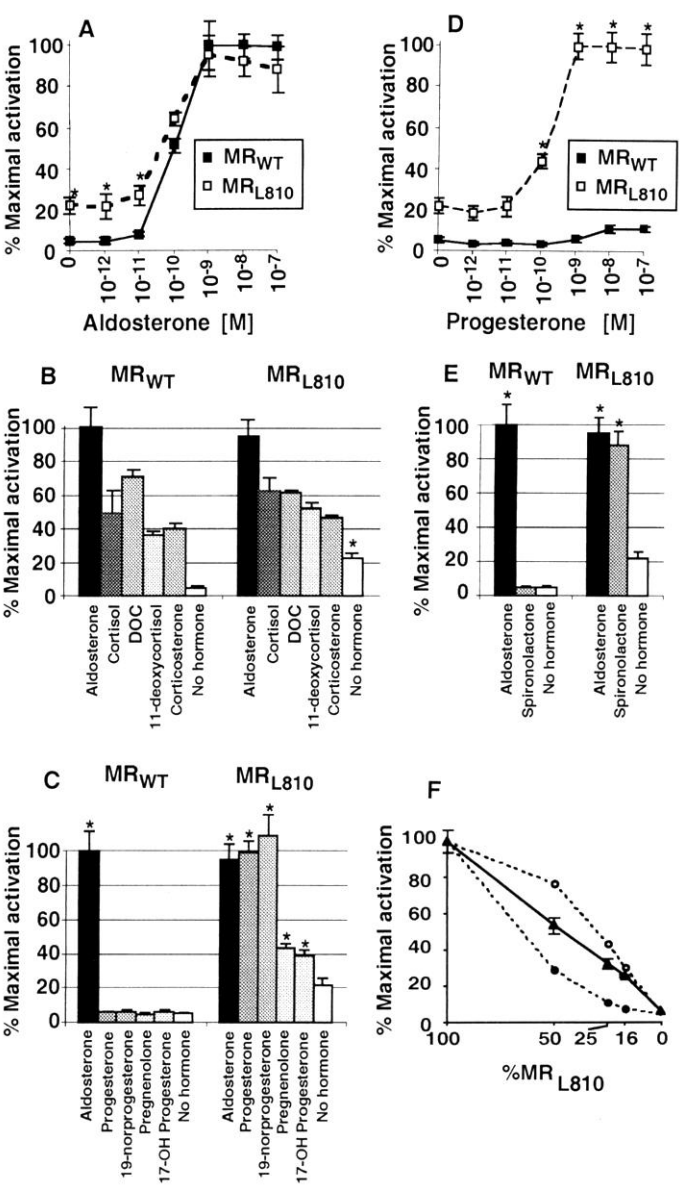

Fig. 2. Transcriptional activation by MR_{WT} and MR_{L810}. The ability of MR_{WT} and MR_{L810} to induce luciferase expressed under control of the MMTV promoter was assessed in Cos-7 cells in the absence or presence of the indicated steroids (*8*). Luciferase activity is expressed as the percentage of maximal induction of MR_{WT} by aldosterone. In (B), (C), (E), and (F), all steroids were assayed at 1 nM. The Mann-Whitney U test was used to compare the significance of differences between groups (in all cases, similar significance was obtained with the two-tailed Student's *t* test). **(A)** Dose-response curve for induction of luciferase by MR_{WT} and MR_{L810} in response to aldosterone (*$P < 0.001$ MR_{L810} versus MR_{WT}). **(B)** Luciferase induction in response to 21-hydroxylated steroids (DOC, 11-deoxycorticosterone; *$P < 0.001$ MR_{L810} versus MR_{WT}). **(C)** Luciferase induction in response to progesterone derivatives (*$P < 0.001$ versus vehicle control). **(D)** Dose-response curve for induction of luciferase by MR_{WT} and MR_{L810} in response to progesterone (*$P < 0.001$ versus vehicle control). **(E)** Luciferase induction in response to spironolactone (*$P < 0.001$ versus vehicle). **(F)** Progesterone-induced luciferase activity in cells coexpressing varying ratios of MR_{WT} and MR_{L810}. Cells were transfected with a constant amount of receptor plasmid (2 μg), with the proportion of MR_{WT} and MR_{L810} varying as indicated. Luciferase induction in response to progesterone (▲) is shown and compared to theoretical curves for completely active (○) and completely inactive (●) heterodimers.

21-hydroxyl group. How does MR_{L810} dispense with this requirement? Ligand binding induces a number of conformational changes, including the bending of helix 3, which in conjunction with helix 12 forms a surface for coactivator binding (*10*). We propose that the conserved helix 5–helix 3 interaction is important for helix 3 bending and receptor activation. In MR, the inability of S810 to interact with A773 suggests that helix 3 bending is achieved by other means. Biochemical studies indicate that N770 interacts with the 21-hydroxyl group of aldosterone (*14*). We propose that this steroid–helix 3 interaction achieves the bending of helix 3 necessary for receptor activation. That steroids lacking a 21-hydroxyl group bind but cannot activate MR_{WT} supports the key role of this interaction. The demonstration that the steroid 21-hydroxyl group becomes dispensable when the helix 5–helix 3 interaction is re-created in MR_{L810} provides evidence that these two interactions are interchangeable for receptor activation. The phenotype of patients harboring this mutation indicates the biological significance of this effect in vivo. Interference with this interaction could provide a general approach to receptor antagonism.

Why MR_{L810} shows constitutive activity, whereas other nuclear receptors with similar helix 5–helix 3 interactions do not, is of interest. One possibility is that formation of an active conformation is aided by an endogenous ligand. In this regard, constitutive activity of an RXR mutant has recently been attributed to binding by an endogenous fatty acid (*18*).

Finally, although the distal nephron is recognized as the major site of action of mineralocorticoids, expression of MR in hippocampus, heart, and endothelium has suggested extrarenal activity (*19*). The early development of congestive heart failure in this kindred raises the question of whether MR_{L810} might contribute to additional clinical effects due to expression in other tissues.

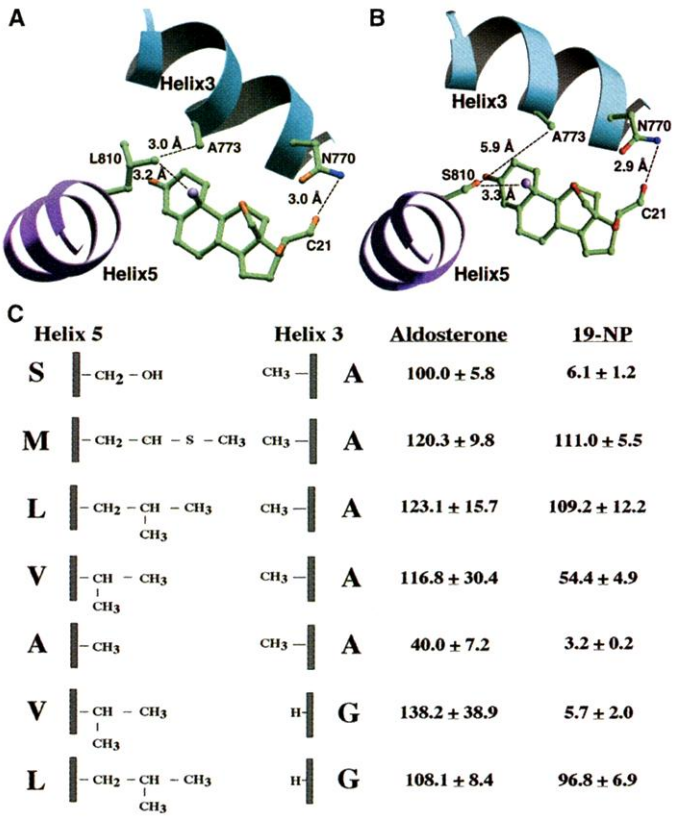

Fig. 3. Helix 3–helix 5 interaction in progesterone-mediated activation of MR_{L810}. (**A**) Structural model of a portion of the HBD of MR_{L810} bound to aldosterone. Based on the crystal structure of the progesterone receptor (*13*), a model of the MR LBD was created by substituting MR-specific residues in the ligand-binding cavity for their corresponding residues in PR (*15*). The side chain of L810 lies in sufficiently close proximity to A773 and the C19 methyl group of the steroid to form van der Waals interactions. (**B**) Model of MR_{WT}. The side chain of S810 does not interact with A773. (**C**) Activity of MRs with various amino acid substitutions at residues 810 and 773. Mutant receptors containing the indicated substitutions at positions 810 in helix 5 and 773 in helix 3 were tested for their ability to induce luciferase activity in the presence of 1 nM aldosterone or 19-NP. The length of each side chain is approximated. Each data point represents the mean of nine independent transfections and is expressed as the mean ± SEM of the percentage of luciferase induction by MR_{WT} in response to aldosterone. Abbreviations for the amino acid residues are as follows: A, Ala; G, Gly; L, Leu; M, Met; S, Ser; and V, Val.

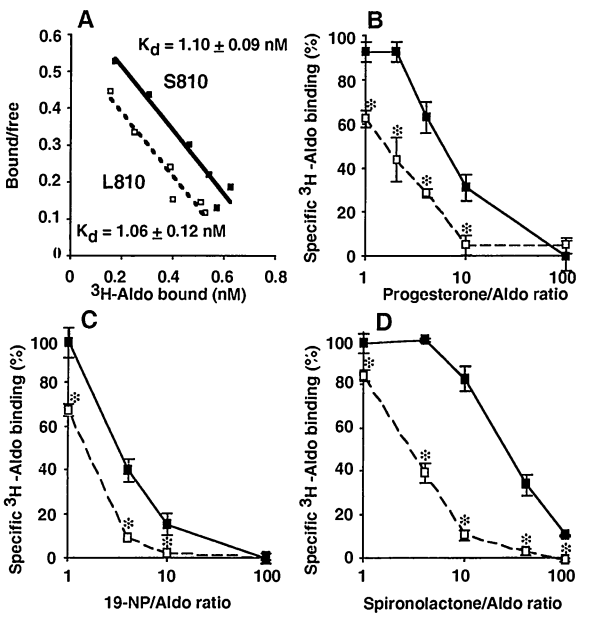

Fig. 4. MR_{L810} has increased affinity for progesterone, 19-NP, and spironolactone. (**A**) Scatchard analysis of the binding of [^{3}H]aldosterone in extracts expressing MR_{WT} (■) or MR_{L810} (□). Each data point was assayed in quadruplicate; one representative set of data for binding to MR_{WT} and MR_{L810} is shown, and the mean K_d's are indicated. (**B** to **D**) Competition of varying concentrations of the indicated unlabeled steroids and 5 nM [^{3}H]aldosterone for binding to MR_{WT} and MR_{L810} in Cos-7 cell extracts. Each data point represents the mean ± SEM of at least three independent experiments. (*) Significant differences between binding of MR_{WT} and MR_{L810}; all differences are significant at $P < 0.001$.

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4. Seventy-five index cases with early onset of severe hypertension, many with suppressed renin and/or aldosterone levels, were screened for mutation by single-stranded conformational polymorphism (SSCP) analysis of all coding regions of *MR* (*3*). Identified variants were subjected to DNA sequence analysis (*3*). All clinical studies were approved by the Yale Human Investigation Committee.
5. Sequence alignment was performed with the Megalign Clustal method software (DNAStar).
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7. Lod scores were calculated with the LINKAGE program (*20*), specifying early onset of severe hypertension as an autosomal dominant trait with complete penetrance, a phenocopy rate of 0.001, and a mutant allele frequency of 0.0001.
8. The plasmid $pRShMR_{NX}$ (*21*) encodes MR expressed from the Rous sarcoma virus long terminal repeat promoter. Site-specific mutations were generated in $pRShMR_{NX}$ by using the Quikchange procedure (Stratagene) with the desired mutagenic primers. Each resulting mutant *MR* gene was sequenced in its entirety to ensure that no undesired mutations were introduced. The plasmid pMTV-Luc (*21*) encodes luciferase under control of the MR-sensitive MMTV promoter. pSV2 (Promega) encodes β-galactosidase (β-Gal) from the SV40 early promoter. Cos-7 cells were cultured in Dulbecco's minimal essential medium (DMEM; Gibco-BRL) supplemented with 10%

heat-inactivated fetal calf serum (FCS). On the night before transfection, cells were plated at 2×10^5 cells per well. Cells in each well were transfected with 2 μg of pMTV-Luc, 2 μg of either MR_{WT} or MR_{L810}, and 0.05 μg of pSV2. Transfection was performed with cationic liposomes (Lipofectamine-Plus; Life Technologies) after which cells were incubated in DMEM supplemented with 10% FCS for 24 hours. Cells were washed, and then serum-free DMEM containing the test steroid was added. The cells were incubated for an additional 16 hours before assay. Luciferase and β-Gal activities were measured as described (*22*). Luciferase activity was normalized to β-Gal activity to correct for transfection efficiency and is expressed as a percentage of the MR_{WT} activity at 1 nM aldosterone. All results are the mean of at least nine independent transfections. For heterodimer experiments, the absolute levels of activity induced by aldosterone with MR_{WT} and MR_{L810} were indistinguishable, and multiple independent preparations of both plasmids yielded indistinguishable results in heterodimer experiments.

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15. The MR_{WT} and MR_{L810} models were generated from the PR crystal structure (*13*) with the Turbo Frodo molecular graphics program (http://afmb.cnrs-mrs.fr/TURBO FRODO). Residues abutting the ligand were altered to match the MR sequence (G773A, M810S, Y941F, and M960L). In addition, the E774G substitution was incorporated to reflect the rotational freedom G774 confers on helix 3 in MR. The geometry was regularized with Turbo Frodo. Coordinates for aldosterone (*23*) were superimposed upon progesterone. Parameters for aldosterone were generated with HIC-Up (*24*). The 21-hydroxyl group was rotated to form a favorable interaction with the N770 side chain (*14*).
16. COS-7 cells were transfected in 100-mm plates with 10 μg of receptor plasmid by using Lipofectamine 2000 (Life Technologies). On the day after transfection, serum-free media was substituted, and the cells were grown for an additional 24 hours. Cells were harvested in 40 mM tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA and lysed by freeze-thaw treatment in hypotonic buffer containing 10 mM tris-HCl (pH 7.8), 10 mM NaCl, 1 mM EDTA, 10 mM Na_2MoO_4, 5 mM dithiothreitol, antipain (5 μg/ml), leupeptin (5 μg/ml), chymostatin (5 μg/ml), pepstatin A (5 μg/ml), and 500 μM phenylmethylsulfonyl fluoride. After centrifugation at 15,000*g* for 15 min, extracts were adjusted to 100 mM NaCl and 5% glycerol (binding buffer). Extracts were incubated overnight with [^{3}H]aldosterone and competitor steroid at 0°C in a total volume of 200 μl, and then incubated with 100 μl of a 50% slurry of hydroxyapatite in binding buffer. Samples were centrifuged, washed twice in binding buffer, and then resuspended in ethanol and prepared for scintillation counting. The value for 100% binding was determined by subtracting the number of counts per minute bound in the presence of 500-fold excess of unlabeled aldosterone from the counts bound in the absence of competitor. Nonspecific binding was determined with a 500-fold excess of unlabeled aldosterone. No specific binding was seen in mock-transfected cells.
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Structure of the Cytoplasmic β Subunit—T1 Assembly of Voltage-Dependent K^+ Channels

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The structure of the cytoplasmic assembly of voltage-dependent K^+ channels was solved by x-ray crystallography at 2.1 angstrom resolution. The assembly includes the cytoplasmic (T1) domain of the integral membrane α subunit together with the oxidoreductase β subunit in a fourfold symmetric $T1_4\beta_4$ complex. An electrophysiological assay showed that this complex is oriented with four T1 domains facing the transmembrane pore and four β subunits facing the cytoplasm. The transmembrane pore communicates with the cytoplasm through lateral, negatively charged openings above the $T1_4\beta_4$ complex. The inactivation peptides of voltage-dependent K^+ channels reach their site of action by entering these openings.

The β subunit of voltage-dependent K^+ channels is a tetramer of oxidoreductase proteins arranged with fourfold rotational symmetry like the integral membrane α subunits (*1*). Each oxidoreductase protein contains an active site with catalytic residues and an NADPH (the reduced form of nicotinamide adenine dinucleotide phosphate) cofactor, but the specific substrate is unknown and the biological function of the β subunit remains a mystery.

Studies of K^+ channel biosynthesis have shown that α and β subunits coassemble in the endoplasmic reticulum and remain together as a permanent complex (*2*, *3*). The idea that a large macromolecular assembly is attached to the intracellular face of voltage-dependent K^+ channels has important implications for channel regulation, but it also raises the question of how the transmembrane pore opens to the cytoplasm. This issue of pore access first arose when the T1 domain, an about 100–amino acid structure on the intracellular side of the first membrane-spanning segment of the α subunit, was found to form a tetrameric ring with a narrow, positively charged central pore (*4*). The small T1 pore diameter and positive charge are inconsistent with functional measurements showing that organic cations such as tetraethylammonium enter the transmembrane pore (*5*). Even a peptide segment from the channel itself (inactivation peptide) is thought to enter the pore to produce inactivation (*6*, *7*). How can entry of these large molecules be reconciled in the setting of a narrow T1 pore? By analyzing the structure and function of the cytoplasmic interface, we resolve this apparent inconsistency and show how the T1 tetramer forms a docking platform for the β subunit without obstructing the transmembrane pathway.

It has been postulated that the intracellular T1 domain interacts with the β subunit (*8*, *9*). We reinforced this idea by showing that removal of the T1 domain, but not the K^+ channel's COOH-terminus, disrupts β subunit association (see below).

Coexpression of the rat β2 subunit (*10*) (residues 36 to 367) and rat Kv1.1 (*11*) T1 domain (residues 1 to 135) in *Sf9* cells yielded a stable complex. The complex was purified and crystallized, and its structure was determined by molecular replacement with the β subunit structure as a search model (*12*). The final model was refined to an R_{free}

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