Characterization of Rat NDRG2 (N-Myc Downstream Regulated Gene 2), a Novel Early Mineralocorticoid-specific Induced Gene*

The early phase of the stimulatory action of aldosterone on sodium reabsorption in tight epithelia involves hormone-regulated genes that remain to be identified. Using a subtractive hybridization technique on isolated renal cortical collecting ducts from rats injected with a physiological dose of aldosterone, we have identified an early response cDNA highly homologous to human and murine NDRG2 (N-Myc downstream regulated gene 2), which consists of four isoforms and belongs to a new family of differentiation-related genes. NDRG2 mRNA was expressed in classical aldosterone target epithelia, and in the kidney, it was specifically located in the collecting duct, the site of aldosterone-regulated sodium absorption. NDRG2 mRNA was increased within 45 min by aldosterone in the kidney and distal colon, whereas it was unaffected in the heart. In the RCC22 collecting duct cell line, NDRG2 mRNA was enhanced as early as 15 min after aldosterone addition by transcription-dependent effects. NDRG2 was induced by aldosterone concentrations as low as $10^{-9}\ \text{M}$, and a maximal effect was observed at $10^{-8}\ \text{M}$. In contrast, the glucocorticoid dexamethasone was ineffective in NDRG2 expression, whereas the glucocorticoid-regulated gene sgk was induced. Taken together, these results indicate that NDRG2 regulation by aldosterone is an early mineralocorticoid-specific effect. Interestingly, NDRG2 is homologous to Drosophila MESK2, a component of the Ras pathway, suggesting that activation of the Ras cascade may play a significant role in mineralocorticoid signaling.

Vertebrates must regulate salt and water excretion to maintain extracellular fluid volume, homeostasis, and blood pressure. The mineralocorticoid hormone aldosterone plays a major role in regulation of sodium absorption in tight epithelia such as the renal collecting duct, the distal colon, and the salivary and sweat glands (1, 2). Its effects are mediated through the mineralocorticoid receptor, a member of the nuclear receptor superfamily that modulates transcription of mostly unknown target genes. This results in stimulation of sodium reabsorption by increasing both the passive luminal entry of sodium into epithelial cells through the amiloride-sensitive epithelial sodium channel (ENaC)\(^1\) and its active extrusion into the blood by the basolateral Na/K-ATPase. However, the hormone-elicited sodium reabsorption occurs before aldosterone-induced transcriptional regulation of ENaC and Na/K-ATPase subunits. Indeed, aldosterone stimulation of transepithelial sodium transport is a complex response (3). An enhanced apical sodium permeability is observed 30–90 min after aldosterone addition, with a maximal effect several hours later. The early phase is thought to involve post-translational regulation such as methylation or phosphorylation of a pre-existing transport machinery. In particular, the activation of "silent" apical sodium channels mainly contributes to the initial increase in apical sodium influx. The late phase of aldosterone response (several hours to days) correlates with enhanced transcription/translation of sodium channel subunits and Na/K-ATPase molecules. The aldosterone-induced regulatory proteins involved in the early phase of hormone response are mostly unknown.

The search for early corticosteroid-induced genes is the subject of intensive investigations. In amphibian A6 cells, induction of ASURs (adrenal steroid-up-regulated RNA) was evidenced. Among them, ASUR5, corresponding to the oncogene K-ras2, stimulates ENaC activity in the Xenopus laevis oocyte expression system (4) and increases sodium transport across A6 cell monolayers (5); however, its role in mammalian cells has not been documented. Attali et al. (6) identified in rat colonic dexamethasone-induced gene (CHIF, for channel-inducing factor) related to other regulatory proteins such as phosholemman and the g-subunit of Na/K-ATPase, but its function remains to be elucidated. Its expression is controlled by aldosterone in the colon, but not in the kidney (7). Substantial recent evidence has pointed to sgk (serum- and glucocorticoid-regulated kinase) as an aldosterone-induced gene in amphibian (8) as well as mammalian (8–12) aldosterone target cells. Induction of sgk mRNA was reported as early as 30 min after treatment with a low dose of aldosterone as well as dexamethasone (10–12). The encoded protein may play a role in mediating early aldosterone effects by stimulating ENaC-mediated sodium transport because coexpression of ENaC and Sgk in X. laevis oocytes increases the channel activity (8–10). Serial analysis of gene expression of aldosterone-induced genes in a mouse collecting duct cell line revealed that a 4-h exposure to aldosterone results in large changes in the transcriptome be-

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The nucleotide sequence(s) reported in this paper has been submitted to the DDBJ/GenBank\textsuperscript{TM}/EBI Data Bank with accession number(s) AJ426424, AJ426425, AJ426426, and AJ426427.

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\(^2\)The abbreviations used are: ENaC, epithelial sodium channel; CCDs, cortical collecting ducts; RACE, rapid amplification of cDNA ends; RPA, RNase protection assay; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PIPES, piperazine-N,N'-bis(2-ethanesulfonic acid); MR, mineralocorticoid receptor; GR, glucocorticoid receptor; 5'-UTR, 5'-untranslated region; MAPR, mitogen-activated protein kinase.
cause 34 transcripts were induced and 29 were repressed (13). Among induced genes, GILZ (glucocorticoid-induced leucine zipper) appears to be an interesting induced candidate in this cellular model.

This study was designed to identify early target genes for aldosterone in native kidney cells and in a physiological context. For this purpose, we used a rat model in which glucocorticoid and mineralocorticoid plasma levels are controlled; such a model has been used for decades to describe early effects of aldosterone in vivo on renal Na⁺ and K⁺ excretion (14). Rats were adrenalectomized, supplemented with glucocorticoid hormone to maintain renal glomerular filtration rate at normal values, and injected acutely with a low dose of aldosterone. Then, a search for early aldosterone-regulated genes was performed by a subtractive hybridization approach conducted on a renal collecting duct preparation.

We have identified a member of the NDRG (N-Myc downstream regulated gene) family, NDRG2, as a putative effector of early effects of aldosterone. We show that the NDRG2 mRNA level was rapidly (45 min) enhanced in vivo by aldosterone in target epithelia such as the distal colon and kidney. In an aldosterone-sensitive cellular model derived from rat cortical collecting duct (RCCD2 cells), the NDRG2 mRNA level was increased as early as 15 min after aldosterone addition via transcription-dependent effects. Dose-dependent effects of aldosterone on NDRG2 expression showed that NDRG2 was induced with 10⁻⁹ M aldosterone, and a plateau was reached at 10⁻⁸ M hormone. In contrast, NDRG2 expression was not affected by the glucocorticoid dexamethasone, whereas the sgk mRNA level was strongly increased. Taken together, these results indicate that NDRG2, a member of a gene family related to cell differentiation, represents a novel mineralocorticoid-specific mediator of early physiological response to aldosterone in target tight epithelia.

**EXPERIMENTAL PROCEDURES**

**Animals**—Experiments were performed on Sprague-Dawley male rats weighing 160–180 g. To select the conditions for differential cloning of early aldosterone-induced genes, urine samples (24 h) were collected after intravenous injection of a unique physiological dose of aldosterone. Rats were adrenalectomized and received a substitutive treatment with control rats (adrenalectomized and dexamethasone-supplemented). Rats were killed 2 h after aldosterone injection. Variations (91 to 104 pg/ml, i.e., 0.2–0.4 nM) were transient because it was no longer apparent 2 h after aldosterone injection.

**Plasma levels of aldosterone from adrenalectomized rats, supplemented with dexamethasone and treated or not with aldosterone, were determined by radioimmunassay (Table I). The plasma aldosterone concentrations from animals used either for subtractive hybridization or for RNAse protection assays are given. 45 min after hormone injection, the plasma aldosterone concentration was increased compared with control rats (adrenalectomized and dexamethasone-supplemented). This rise was within the physiological range of hormone level variations (91–145 pg/ml, i.e., 0.2–0.4 nM) and was transient because it was no longer apparent 2 h after aldosterone injection.

**Cellular Model**—The RCCD2 model has been described (15). RCCD2 cells were cultured in complete medium containing Dulbecco’s modified Eagle’s medium/Ham’s F-12 medium (1:1), 14 mM NaHCO₃, 2 mM glutamine, 5 × 10⁻⁸ M dexamethasone, 6 × 10⁻⁸ M sodium selenite, 5 µg/ml transferrin, 5 µg/ml insulin, 10 ng/ml epidermal growth factor, 10 units/ml penicillin/streptomycin, 2% fetal bovine serum (Invitrogen), and 20 µM Hepes, pH 7.4. For experiments, cells were seeded on Transwell filters (Corning Costar Corp.) previously coated with collagen (Institut J. Boy, Reims, France). Cells were incubated overnight in minimal medium (Dulbecco’s modified Eagle’s medium/Ham’s F-12 medium (1:1), 14 mM NaHCO₃, 2 mM glutamine, 10 units/ml penicillin/streptomycin, and 20 µM Hepes, pH 7.4) and then treated or not with aldosterone (10⁻¹² to 10⁻⁷ M) or dexamethasone (5 × 10⁻⁸ M) for various times. Actinomycin D was from Sigma, and RU486 was a kind gift from Roussel-Uclaf.

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Briefly, kidneys from control or aldosterone-treated rats (eight rats in each group, killed 45 min after intravenous injection of aldosterone or vehicle) were removed, and cortex slices were digested with collagenase (0.75 mg/ml for 1 h at 37 °C; Serva) and then submitted to differential centrifugation on a Percoll gradient (40%). The lowest density band corresponded mainly to CCD fragments (25–40 mg), with minor contamination by glomeruli or proximal tubules. Poly(A) mRNAs were extracted from hormone-treated or control CCDs using oligo(dT)₃₄ covalently bound to magnetic beads (Dynal A/S, Oslo, Norway).

To identify up-regulated sequences, the two pools of mRNA fragments derived from control and aldosterone-treated animals were submitted to PCR-based subtractive hybridization and suppression PCR (PCR-Select™ cDNA subtraction kit, CLONTECH) according to the manufacturer’s protocol. Double-stranded cDNA was synthesized and digested with RsaI. Adaptors were then linked to the aldosterone-treated cDNA pool, and two successive hybridizations followed by extension to fill in ends were performed in the presence of an excess of cDNA without linkers from untreated CCDs. The first PCR amplification using suppression PCR amplified exponentially only differentially expressed sequences. The second PCR amplification allowed us to reduce background levels and further enriched differentially expressed cDNA fragments. “Forward” subtraction was performed when linkers were added to cDNA fragments obtained from hormone-treated CCDs, whereas “reverse” subtraction was performed when linkers were added to cDNA fragments obtained from control CCDs. PCR products were cloned into the pF-Adv vector using the AdvinciTag™ PCR cloning kit (CLONTECH). Differential screening of the subtracted library was performed to eliminate false positives by hybridization with ³²P-labeled probes prepared from forward- and reverse-subtracted cDNAs (PCR-Select™ differential screening kit, CLONTECH). Clones showing signal ratios of >5:1 (forward-subtracted versus reverse-subtracted probes) were further analyzed by DNA sequencing (GENOMEX, Paris, France).

**Cloning of Full-length Rat NDRG2**—The full-length sequence of one
exposed sequence tag (447 bp) was established by screening a rat kidney cDNA library (kindly provided by M. C. Leconte, INSERM U409, Faculté de Médecine Xavier Bichat) by anchored PCR. We performed the first series of PCR with an antisense oligonucleotide (AS1) localized in the sequence of the exposed sequence tag and a sense oligonucleotide (S1) specific to the pGAD/T3 plasmid containing the library. To improve the specificity of the PCR products, we also carried out nested PCR with a second set of oligonucleotides, AS2 and S2. The different oligonucleotides were designed as follows: S1, 5'-CGATGGAT-GAAGATACCCCACC-3'; AS1, 5'-TTAGGAGGAGTGGCCTTCGAG-3'; S2, 5'-AGAGATAGCTAAGGTCTCGG-3'; AS2, 5'-AGGACTTTGAGT-CAGCCTTGGGGG-3'. PCRs were performed in 50 μl of a mixture containing 1X Advantage-2 PCR buffer, 200 μM dNTPs, 50 pmol of each oligonucleotide (S1/AS1 or S2/AS2), 10 ng of rat kidney library, and 1X Advantage-2 polymerase mixture (Clontech) in a 94°C-57°C-72°C-94°C-72°C cycle. Cycles were as follows: 94°C for 5 min and then 94°C for 1 min, 54°C for 1 min, and 72°C for 1 min for 20 cycles. PCR products (~740 bp) were sequenced and used to design an antisense oligonucleotide (GSP1, 5'-AGAGGGTGTGCAACAGGAAGTCT-3') to clone cDNA 5'-ends by rapid amplification with the SMART™ RACE cDNA amplification kit (Clontech). Briefly, first-strand cDNA synthesis was performed with 1 μg of total RNA from rat heart (a tissue strongly expressing NDRG2; see Fig. 4) with the SMART II oligonucleotide and Superscript II reverse transcriptase (Invitrogen) according to the manufacturer's protocol. 5'-RACE/PCRs were realized with GSP1 and Universal Primer Mix oligonucleotides (Clontech) as indicated by the manufacturer's protocol. 5'-RACE products were characterized by sequencing (GENOME Expresses).

In Situ Hybridization—Rat tissues were fixed by perfusion via the aorta with 4% paraformaldehyde in phosphate-buffered saline (130 mM NaCl, 7 mM Na2HPO4, and 3 mM NaH2PO4, pH 7.4). Organs were dehydrated in graded alcohol solutions and embedded in paraffin (Paraplast). Hybridization was then carried out on tissue sections as previously described (17). The 3'-untranslated region of NDRG2 cDNA (nucleotides 1500–1946 of NDRG2b 2) or 18 S RNA (nucleotides 1428–1946 of NDRG2b or 3 mM (18 S RNA) MgCl2, 200 μM dNTPs and 1.25 units of Taq polymerase. The final primer and probe concentrations were 400 and 100 nM, respectively. PCR reagents were from Eurogentec (qPCR core kit). The thermal cycling conditions comprised an initial denaturation step at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. Standard curves were generated using serial dilutions of a purified restriction fragment of NDRG2 (nucleotides 1500–1946 of NDRG2b) or 18 S RNA (nucleotides 1428–1788), covering 5 orders of magnitude and yielding correlation coefficients of at least 0.98 in all experiments. Each standard and sample values were determined in triplicate in three independent experiments. In each experiment, NDRG2 expression under a given experimental condition was calculated relative to the control condition.

Statistical Analysis—Data are expressed as means ± S.E. (n = number of animals). Statistical analysis was performed using Student’s t test after analysis of variance.

RESULTS

Cloning of Rat NDRG2, a Member of the NDRG Gene Family—PCR-based subtractive hybridization was used to establish a library of cDNAs representing mRNAs rapidly (45 min) up-regulated by a physiological dose of aldosterone in isolated rat CCDs. Selected clones were sequenced and analyzed for sequence homology using the BLAST program. About 50% of them corresponded to genes involved in oxidative metabolism (cytochrome c oxidase subunits) and the cytoskeleton (actin). One of the clones (clone 2, 447 bp) that was homologous to expressed sequence tag 239096 (DBJ/GenBank™/EBI accession number AI410803) was then subjected to further studies.

The full-length sequence of clone 2 was obtained using RACE. Four different cDNAs were isolated. The cDNA sequences of rat NDRG2a, NDRG2b, NDRG2c, and NDRG2d have been deposited in the DBJ/GenBank™/EBI Data Bank under accession numbers AJ426424, AJ426425, AJ426426, and AJ426427, respectively. They share a common sequence over 1927 nucleotides and were shown to correspond to different mRNA isoforms. A new BLAST search revealed ~90% sequence homology to mouse and human NDRG2 cDNAs, mem-

### Table 1

| Series 1 | Control | Aldo (45 min) | Aldo (2 h) |
|----------|---------|--------------|------------|
| Aldo (pg/ml) | < 5 (8) | 91 ± 11.9 (8) | 6 ± 4.5 (3) |
| Series 2 | Control | Aldo (45 min) | Aldo (2 h) |
| 6 ± 4.5 (3) | 145 ± 19.9 (3) | 7.1 ± 3.4 (3) |
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NDRG2 Is Rapidly Induced by a Physiological Dose of Aldosterone in Hormone-sensitive Epithelia—To examine aldosterone effects on NDRG2 expression, we performed RPAs in the kidney and distal colon from adrenalectomized and glucocorticoid-supplemented rats injected with aldosterone or vehicle (i.e. control condition). We were also interested in non-epithelial tissues such as the heart in which the hormone exerts effects by unknown mechanisms. Interestingly, aldosterone displays differential tissue-specific effects on NDRG2 mRNA expression. Fig. 7A illustrates the results from a typical RPA obtained with the NDRG2 probe hybridized with kidney RNA from control and aldosterone-treated rats for 45 min and 2 h. Fig. 7 (B–D) illustrates NDRG2 expression normalized to GAPDH and expressed as a percentage of controls in the kidney, distal colon, and heart, respectively. In the kidney, aldosterone induced a significant increase in NDRG2 mRNA expression 45 min after injection, and this increase was maintained at 2 h. A similar induction was observed in the distal colon, where NDRG2 expression was increased 45 min and 2 h after aldosterone injection. In contrast, NDRG2 mRNA expression was not modulated by aldosterone in the heart 45 min or 2 h after hormonal injection.

The mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR) are coexpressed in aldosterone target cells (2, 20), and aldosterone binds to both type of corticosteroid receptors (with a higher affinity for the MR than the GR). To address the question of the specificity of aldosterone effects on NDRG2 expression and to better characterize these effects, RPA and quantitative real-time PCR experiments were carried out in the rat cortical collecting duct cell line RCCD2. Characterization of the RCCD2 cell line has been recently reported (15). This cellular model has maintained many characteristics of the CCDs, including aldosterone-induced sodium transport. It expresses the MR and the GR, and low concentrations of aldosterone (10⁻¹⁰ to 10⁻⁸ m) induce an increase in the short-circuit...
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The aldosterone effects on NDRG2 were examined by real-time PCR (for NDRG2) and RPA (for sgk) on the same samples (Fig. 8B). Although the NDRG2 mRNA level was not modified by dexamethasone, sgk was strongly induced, and this effect was abolished in the presence of the GR antagonist RU486. These results indicate that although the GR is functional in RCCD2 cells, the glucocorticoid dexamethasone does not regulate NDRG2 expression. In addition, the dose dependence of the aldosterone effects on NDRG2 shows that NDRG2 was induced at physiological concentrations because the NDRG2 mRNA level was increased by $10^{-8}$ M aldosterone, and a plateau was obtained with $10^{-5}$ M hormone (Fig. 8C). Taken together, these results suggest that NDRG2 stimulation by aldosterone is a mineralocorticoid-specific response.

Fig. 9A shows that NDRG2 is an early induced gene because its mRNA level was significantly increased as early as 15 min after aldosterone addition. This increase was still present at 1 h, and a return to the basal level was observed after 2 h. We examined whether transcription was required in the NDRG2 response to aldosterone using the transcription inhibitor actinomycin D (Fig. 9B). Preincubation of RCCD2 cells with actinomycin D 60 min before aldosterone addition prevented the rise in the NDRG2 mRNA level observed in the presence of aldosterone alone. Thus, NDRG2 regulation by aldosterone is transcription-dependent.

**DISCUSSION**

Early aldosterone effects on transepithelial sodium transport in tight epithelia are mediated by hormone-induced regulatory proteins, which are mostly unknown. The main finding of this study is that a physiological dose of aldosterone rapidly increases the mRNA level of NDRG2, a differentiation-related gene, in rat epithelial cells via a mineralocorticoid-specific effect.

In our search for rapid aldosterone-induced mRNAs, we have cloned and characterized four different cDNAs for rat NDRG2, the homolog of mouse and human NDRG2. These cDNAs code for the same putative NDRG2 protein except for the presence or absence of 14 residues in the N-terminal region, which are encoded by a 42-bp insertion (the calculated molecular mass is either 39.3 or 40.8 kDa). Comparison of the cDNAs with sequences contained in the Human Genome Database indicates that this insertion corresponds to the inclusion of exon 3 of the human NDRG2 gene, suggesting an alternative splicing event. These two variants were also identified in the rat, as illustrated in Fig. 2. Interestingly, besides NDRG2b1 and NDRG2b2, which share a common 5'-untranslated region (5'-UTR), we have identified two new NDRG2 variants, NDRG2a1 and NDRG2a2, which contain a new 5'-UTR of 87 nucleotides. Sequences highly homologous to the two rat NDRG2 5'-UTRs were found on different exons in the human NDRG2 gene, indicating that they might correspond to alternative 5'-untranslated exons. This genomic organization strongly suggests the presence of alternative promoters that could direct expression of NDRG2 in a tissue-specific and developmentally regulated manner.

It is noteworthy that a glucocorticoid-responsive element half-site (TGGTCT) was found in the human...
**NDRG2** promotor, corresponding to the sequence flanking the **NDRG2** 5'-UTR.

**NDRG2** belongs to a new family of differentiation-related genes, the **NDRG** family. This family includes four recently identified related members: **NDRG1**–4 (22). **NDRG1** was first seen in various tissues in different species (23–28). Two other members of the family were then identified in the mouse: **Ndr2** and **Ndr3** (29). A recent report by Zhou et al. (22) documents the human **NDRG** gene family with characterization of three isoforms of **NDRG4**, the equivalent of rat **BDM1** (30). Human **NDRG** members are highly homologous except in their C- and N-terminal regions. They exhibit distinct patterns of expression during development and adult life. **NDRG1** is a widely expressed gene; **NDRG2** and **NDRG3** are essentially expressed in the brain, heart, skeletal muscle, and kidney, and **NDRG4** expression is restricted to the brain and heart. This variable tissue-specific expression in the **NDRG** gene family suggests that each member may exert a particular role in different organs.

The functional role of **NDRG** genes remains to be established. Phylogenetic analysis revealed that **NDRG** genes are highly conserved in plants, invertebrates, and mammals, sug-
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Fig. 7. Aldosterone effects on NDRG2 mRNA expression in the kidney, distal colon, and heart. A, RPA was performed in kidneys from control rats (Control; i.e., adrenatecomized and supplemented with dexamethasone) or from rats injected with a unique physiological dose of aldosterone (0.2 µg/100 g of body weight) 45 min (Aldo 45 min) or 2 h (Aldo 2h) before they were killed. Total RNA from kidney and yeast tRNA (40 µg/lane) were hybridized with NDRG2- and GAPDH-specific probes. Each lane corresponds to a different rat. MW, molecular weight markers. The protected fragments have expected sizes of 447 nucleotides for NDRG2 and 164 nucleotides for GAPDH. No background signal was detected with tRNA. B-D, quantification of NDRG2 mRNA expression in the kidney, distal colon, and heart, respectively, from control and aldosterone-treated rats was performed using an Instant Imager. NDRG2 mRNA expression was normalized to the internal standard GAPDH and expressed as a percentage of the control condition (without hormone). Values are means ± S.E. of three independent experiments (n = three rats in each group). *p < 0.05; §p < 0.025 (aldosterone versus control).

NDRG2 was evident only in epithelial target cells (kidney and distal colon) and not in the heart. This suggests that the early aldosterone response may be mediated by different mechanisms or effectors among target cells, as expected from their distinct functional characteristics. It is noteworthy that a similar lack of effect of the hormone on Sgk expression in the heart has been recently reported (12). The aldosterone-elicited increases in NDRG2 mRNA in the kidney and colon are relatively moderate (45–65%). Aldosterone-related changes of such limited amplitude have been previously described for transcripts encoding different subunits of the sodium channel (18, 38, 39) or the Na/K-ATPase (17, 18). Such effects may be amplified at the protein level. It is also likely that other regulatory mechanisms or post-transcriptional effects of the hormone may be involved in the physiological response to aldosterone. We also provide evidence that the aldosterone-mediated increase in NDRG2 mRNA is transcription-dependent in the RCCD2 cell line (Fig. 9B). This transcription effect is rapid, as it was observed as early as 15 min after aldosterone addition (Fig. 9A). Such rapid transcriptional effects have been previously reported for Sgk induction by aldosterone.

Aldosterone target cells coexpress the MR and the GR (20). Aldosterone binds to the MR with high affinity (Kd = 10^{-10} to 10^{-9} M) and also to the GR with a lower affinity (Kd = 10^{-9} to 10^{-8} M). Thus, low concentrations of aldosterone (<10^{-9} M) will bind to the MR, whereas higher concentrations of the hormone (>10^{-8} M) will occupy both the MR and the GR. These two receptors regulate transcription of different (but partially overlapping) networks of genes. For this reason, it has often been difficult to distinguish between MR- and GR-mediated effects, especially because early receptor-specific responses are largely unknown. In many studies devoted to the search for early aldosterone-regulated genes, high doses of the hormone were used (4, 8); and thus, the specificity of the response was difficult to evaluate. The experiments performed in the RCCD2 cell line

going important functions of this gene family (22). NDRG1 was initially designated as RTP (reducing agent- and fungamycin-responsive protein), and its expression was shown to be regulated by homocysteine in cultured human umbilical vein endothelial cells (23). RTP was then isolated from various human and murine tissues (and referred to as Drg1, rit42, and Cap43 for the human homolog (24–26) and TDD5 and Ndr1 for the murine homolog (27, 28)). NDRG1 expression is regulated by chemical stimulation, which may impose a stress on the cells (23, 26, 31). Accumulated data suggest that NDRG1 plays a role in growth arrest and cell differentiation and could act as a signaling protein shuttling between the cytoplasm and the nucleus. Indeed, NDRG1 expression is up-regulated during cell differentiation (24, 31–34) and repressed during cell transformation (24, 25) and by N-myc or c-myc (28), known to inhibit terminal differentiation and to stimulate cell proliferation. Moreover, anti-oncogenic effects have been proposed: forced expression of NDRG1 in tumor cells reduces cell growth, increases cell differentiation, and reduces the metastatic potency of the cells (25, 35). Interestingly, it has recently been shown that a mutation of the NDRG1 gene is causative for hereditary motor and sensory neuropathy-lom, suggesting a role of NDRG1 in the peripheral nervous system (36). Concerning the other members of the family, no information exists on NDRG2, NDRG3, and NDRG4 functions. Their pattern of expression is overlapping networks of genes. For this reason, it has often been difficult to distinguish between MR- and GR-mediated effects, especially because early receptor-specific responses are largely unknown. In many studies devoted to the search for early aldosterone-regulated genes, high doses of the hormone were used (4, 8); thus, the specificity of the response was difficult to evaluate. The experiments performed in the RCCD2 cell line
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All experiments were performed on 35 μg of total RNA with NDRG2 and GAPDH probes, and signal quantification was then realized using an Instant Imager. NDRG2 mRNA expression was normalized to the internal standard GAPDH and expressed as a percentage of the control condition. Values are means ± S.E. of four independent experiments, with each experiment performed in triplicate.

Fig. 8. Differential effects of aldosterone and dexamethasone on NDRG2 and sgk mRNA expression in rat RCCD2 cells. A, cells grown on filters were incubated without (Control) or with 10^{-6} M aldosterone (Aldo) or 5 × 10^{-8} M dexamethasone (Dex) for 45 min or 2 h. RPA experiments were performed on 35 μg of total RNA with NDRG2 and GAPDH probes, and signal quantification was then realized using an Instant Imager. NDRG2 mRNA expression was normalized to the internal standard GAPDH and expressed as a percentage of the control condition. Values are means ± S.E. of four independent experiments, with each experiment performed in triplicate. B, shown is NDRG2 and sgk expression after dexamethasone treatment in RCCD2 cells. RCCD2 cells were grown on filters and incubated with dexamethasone (5 × 10^{-8} M) in the absence or presence of RU486 (10^{-5} M) for 45 min. NDRG2 was measured by quantitative real-time PCR, normalized to the internal standard 18 S RNA, and expressed as a percentage of the control condition (in the absence of any treatment). sgk was evaluated by RPA, normalized to GAPDH, and expressed as a percentage of the control condition. NDRG2 and sgk were determined on the same filters, and values are means ± S.E. of three independent experiments. C, shown is the dose dependence of aldosterone effects on NDRG2 mRNA. RCCD2 cells grown on filters were incubated with or without various concentrations of aldosterone (10^{-11} to 10^{-7} M) for 30 min. NDRG2 mRNA was measured by quantitative real-time PCR, normalized to 18 S RNA, and expressed as a percentage of the control condition (in the absence of aldosterone). Values are means ± S.E. of three independent experiments, with each experiment performed in triplicate. P < 0.025; **, P < 0.010 (aldosterone versus control).

allowed us to progress in the characterization of aldosterone effects on NDRG2 expression. Here, we have demonstrated that the glucocorticoid dexamethasone did not affect NDRG2 expression, although the GR was functional in the RCCD2 cells because sgk transcripts were induced by dexamethasone (Fig. 8, A and B). Although dexamethasone binds to the MR with an affinity close to that of aldosterone, this glucocorticoid hormone appears to be poorly efficient in promoting MR transactivation activity (40). Thus, the dexamethasone-induced increase in sgk mRNA is likely a GR-mediated (not MR-mediated) event. We also showed that NDRG2 mRNA was up-regulated by low doses of aldosterone (Fig. 8C). The absence of NDRG2 regulation by dexamethasone and the capacity of physiological concentrations of aldosterone to induce NDRG2 strongly suggest that the aldosterone-induced modulation of NDRG2 is a mineralocorticoid-specific response. Thus, this is the first characterization of an early mineralocorticoid-specific regulated gene in epithelial cells.

The existence of this family of genes and their conservation through evolution suggest an important biological role; however, their involvement in cell function and disease development remains to be elucidated. It is noteworthy that in A6 epithelia, aldosterone down-regulates the mRNAs of the proto-oncogenes c-myc, c-jun, and c-fos, which play a major role in stimulation of cell growth and inhibition of terminal differentiation (41). These facts may be related to our results demonstrating that aldosterone up-regulates the mRNA of a gene that belongs to a family referred to as “N-Myc downstream regulated genes,” putatively involved in growth arrest and induction of cell differentiation. Along this line, a recent study reports that aldosterone favors very early adipocyte differentiation in a brown adipocyte cell line (derived from a hibernoma of transgenic mice overexpressing the large T antigen of SV40 under the control of the proximal human MR promoter) (42). Hence, new aspects of the pleiotropic action of the hormone are emerging, especially in terms of regulation of target cell differentiation status. The ability of aldosterone to favor cell differentiation can be understood in view of the fact that enhanced transepithelial sodium transport capacity requires multiple cellular processes occurring only in highly differentiated cells.

Interestingly, a search for known structural or functional domains within the deduced NDRG2 amino acid sequence re-
revealed homology to the α/β-hydrolase fold (InterPro entry IPR000073), which is common to a number of hydrolytic enzymes of widely differing phylogenetic origin and catalytic function, suggesting that NDRG2 may have an enzymatic function. It is noteworthy that the human NDRG2 gene has 34% identity to MESK2 (misexpression suppressor of dominant-negative KSR (kinase suppressor of Ras)), a gene recently identified in a misexpression screen conducted in Drosophila to search for genes that modulate the Ras1 signaling pathway (43). MESK2 may represent a new component of the Ras pathway or of other signaling pathways that can modulate signaling by Ras. The MAPK cascade is a central signaling module through which the small GTPase Ras, in response to diverse stimuli, exerts control over cell growth, cell differentiation, or apoptosis (44, 45). Activated MAPK (via phosphorylation processes) regulates the activities of cytoplasmic and nuclear tar-

**Fig. 9. Time course of aldosterone effects on NDRG2 expression in rat RCCD2 cells.** A, cells grown on filters were incubated with or without 10⁻⁸ M aldosterone for various times. Expression of NDRG2 was studied by quantitative real-time PCR. The total RNA concentration was measured using the ultrasensitive fluorescent nucleic acid stain RiboGreen, which allows an accurate determination of RNA concentration and standardization between samples. Relative induction of NDRG2 was expressed as a percentage of the control condition (in the absence of aldosterone). Values are means ± S.E. of three independent experiments, with each experiment performed in triplicate. *, *p < 0.025; **, *p < 0.005; ***, *p < 0.001 (aldosterone versus control). B, shown is the influence of actinomycin D on NDRG2 induction by aldosterone in RCCD2 cells. Cells grown on filters were preincubated with or without actinomycin D (Actino; 2 × 10⁻⁶ M) for 1 h and then treated or not with aldosterone (Aldo; 10⁻⁸ M) for 30 min (in the presence or absence of actinomycin D). NDRG2 was measured by quantitative real-time PCR, normalized by 18 S RNA, and expressed as a percentage of the control condition. Values are means ± S.E. of three independent experiments, with each experiment performed in triplicate. **, *p < 0.005 (aldosterone versus control).
gets, which in turn lead to specific cellular responses. It is interesting to note that two recently identified early corticosteroid-induced regulatory proteins, K-Ras2 and Sgk, are directly or indirectly part of the Ras signaling pathway. Indeed, Sgk is a target of 3-phosphoinositide-dependent kinase-1, which itself is activated by the products of phosphatidylinositol 3-kinase. Because Ras is an upstream regulator of phosphatidylinositol 3-kinase, in some circumstances, Sgk activity may depend on Ras activity. Although K-Ras2 mRNA was not found in rat collecting duct, other members of the Ras family may be involved in aldosterone action in mammalian cells. Thus, the regulatory network controlled by aldosterone is highly complex. Its early response involves induction of a number of effectors that are the site of convergence of different intracellular cascades and that contribute in synergy to sodium transport regulation. The elucidation of these signaling pathways should bring new insights into the understanding of corticosteroid hormone action in epithelia and its dysfunctions in diseases.

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