(NDRG2) Stimulates Amiloride-sensitive Na\textsuperscript{+} Currents in Xenopus laevis Oocytes and Fisher Rat Thyroid Cells

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Regulation of the epithelial sodium channel (ENaC)\textsuperscript{2} is highly complex and may involve several aldosterone-induced regulatory proteins. The N-Myc downstream-regulated gene 2 (NDRG2) has been identified as an early aldosterone-induced gene. Therefore, we hypothesized that NDRG2 may affect ENaC function. To test this hypothesis we measured the amiloride-sensitive current (\(\Delta I_{\text{ami}}\)) whole cell current in Xenopus laevis oocytes expressing ENaC alone or co-expressing ENaC and NDRG2. Co-expression of NDRG2 significantly increased \(\Delta I_{\text{ami}}\) in some, but not, all batches of oocytes tested. An inhibitory effect of NDRG2 was never observed. Using a chemiluminescence assay we demonstrated that the NDRG2-induced increase in ENaC currents was accompanied by a similar increase in channel surface expression. The stimulatory effect of NDRG2 was preserved in oocytes maintained in a low sodium bath solution to prevent sodium feedback inhibition. These findings suggest that the stimulatory effect of NDRG2 is independent of sodium feedback regulation. Furthermore, the stimulatory effect of NDRG2 on ENaC was at least in part additive to that of Sgk1. A short isoform of NDRG2 also stimulated \(\Delta I_{\text{ami}}\). Overexpression of NDRG2 and ENaC in Fisher rat thyroid cells confirmed the stimulatory effect of NDRG2 on ENaC-mediated short-circuit current (\(I_{\text{sc-am}}\)). In addition, small interference RNA against NDRG2 largely reduced \(I_{\text{sc-am}}\) in Fisher rat thyroid cells. Our results indicate that NDRG2 is a likely candidate to contribute to aldosterone-mediated ENaC regulation.

The epithelial sodium channel (ENaC)\textsuperscript{2} consists of three subunits (\(\alpha, \beta, \gamma\)) and is localized in the apical membranes of sodium absorbing epithelia like the aldosterone-sensitive distal nephron, respiratory epithelia, distal colon, sweat, and salivary ducts. It is the rate-limiting step for sodium absorption in these epithelia and plays a major role in the maintenance of body sodium balance (1, 2). The appropriate regulation of ENaC is critically important for the long term control of arterial blood pressure (3). This is evidenced by gain-of-function mutations of ENaC causing Liddle syndrome (pseudohyperaldosteronism), a hereditary form of severe arterial hypertension (4). In contrast, loss-of-function mutations of ENaC cause pseudohypoaldosteronism type 1, a salt-wasting syndrome with low blood pressure (5).

The main hormonal regulator of ENaC is aldosterone (1, 2). It is well accepted that aldosterone increases ENaC expression in the apical membrane of principal cells in the aldosterone-sensitive distal nephron (6–9). In addition an increase in channel open probability and the activation of near-silent channels are mechanisms involved in ENaC activation by aldosterone (1, 10). The aldosterone response has a characteristic time course. During the so-called “early response” (1.5–3 h) the predominant effect is an activation of pre-existing Na\textsuperscript{+} channels and pumps. This is followed by a “late response” (6–24 h) characterized by the transcriptional and translational up-regulation of the expression of ENaC subunits and the Na\textsuperscript{+}/K\textsuperscript{+}-ATPase (1). In particular, during the early response aldosterone-induced regulatory proteins are thought to contribute to ENaC activation. For example, the small G-protein K-Ras2 (11) and the serum- and glucocorticoid-inducible kinase (Sgk1) (12–14) have been identified as early aldosterone-induced genes and are believed to be ENaC-regulatory proteins. K-Ras2 has been reported to activate ENaC via the phosphatidylinositol 3-kinase pathway (15). Sgk1 has been shown to phosphorylate the ENaC-regulatory protein Nedd4-2, thereby rendering it unable to bind to and ubiquitinate ENaC, which results in a decreased retrieval rate of the channel and hence an increase in its surface expression (16–18). Channel activation by Sgk1, however, is likely to involve more than one mechanism (12), including a critical phosphorylation site in the C terminus of \(\alpha\)-ENaC (19).

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2 The abbreviations used are: ENaC, epithelial amiloride-sensitive sodium channel; \(\Delta I_{\text{ami}}\), amiloride-sensitive current; NDRG2, N-Myc downstream-regulated gene 2; Sgk1, serum- and glucocorticoid-inducible kinase; FRT, Fisher rat thyroid; \(I_{\text{sc-am}}\), amiloride-sensitive short-circuit current; Nedd4-2, neuronal precursor cell-expressed developmentally down-regulated 4-2; RCCD, rat kidney cortical collecting duct cell line; wt, wild-type; MBS, modified Barth’s solution; \(R_{\text{te}}\), transepithelial resistance; \(V_{\text{te}}\), transepithelial voltage; PBS, phosphate-buffered saline; \(I/V\), current-voltage; N4WBP5A, Nedd4 WW-domain binding protein 5A; MESP2, misexpression suppressor of dominant-negative KSR (kinase suppressor of Ras).
Looking for additional early induced target genes of aldosterone, Bouklkron and co-workers recently identified a member of the N-Myc downstream-regulated gene (NDRG) family, NDRG2, as a putative effector of early effects of aldosterone in native rat kidney cells (20). Four members of the NDRG family are known thus far, but the physiological function of these proteins is still unknown. Bouklkron et al. (20) could identify four different isoforms of rat NDRG2 CDNA, which differ either in the 5′-untranslated sequence or in the coding sequence or both, and encode for a longer 371-amino acid NDRG2 protein and a shorter 357-amino acid NDRG2 protein, referred to here as NDRG2-sh. NDRG2 mRNA is expressed in human kidney (21), rat distal colon and kidney (20), and throughout the tubules in the developing (22, 23) and adult mouse kidney (23), but not in the glomerulus. In situ hybridization of rat kidney revealed NDRG2 expression mainly in the distal tubule, the cortical and outer medullary collecting duct, and some expression also in the proximal convoluted tubule (20, 24). NDRG2 mRNA levels were rapidly increased in vivo within 45 min of aldosterone treatment in the kidney and distal colon of adrenalectomized adult rats and even faster after 15 min in cultured rat kidney systems co-expression of NDRG2 and ENaC resulted in a stimulatory effect of NDRG2 on ENaC currents was paralleled by an increase in ENaC surface expression. In FRT cells siRNA against NDRG2 largely abolished ENaC-mediated Na+ currents, which suggests that the presence of NDRG2 is important for ENaC function.

**EXPERIMENTAL PROCEDURES**

**Plasmids**—Full-length cDNAs encoding for the longer and the shorter isoform of rat NDRG2 are in pSDeasyBS. Plasmids containing full-length cDNAs encoding for wild-type rat α-, β-, and γ-ENaC (pGEM-HE) (27) and for rat β-FLAG-ENaC (pSD5) (28) were kindly provided by Bernard C. Rossier and Laurent Schild (Lausanne, Switzerland). Plasmids encoding constitutively active human Sgk1-SD (pGEM-HJ) (29, 30) were kindly provided by Laurent Schild (Lausanne, Switzerland). Plasmids containing cDNAs encoding for NDRG2 and ENaC, NDRG2, or Sgk1 (injected amounts of cRNA are given under “Results” or in the figure legends). The cRNAs were dissolved in RNase-free water, and the total volume injected was 46 nl per oocyte. Injected oocytes were stored at 19°C in modified Barth’s solution (MBS) either with high Na+ (NaCl 85, KCl 1, NaHCO3 2.4, Ca(NO3)2 0.3, CaCl2 0.4, MgSO4 0.8, HEPES 10, pH 7.4, with Tris) or low Na+ (NaCl 1, KCl 40, NaHCO3 2.4, Ca(NO3)2 0.3, CaCl2 0.4, MgSO4 0.8, HEPES 5, pH 7.4, with Tris) at a rate of 2–3 nl/min at room temperature. Voltage clamp experiments were performed using an OC-725C amplifier (Warner Instruments Corp., Hamden, CT) interfaced via an LIH-1600 (HEKA, Lambrecht, Germany) to a PC running PULSE software (HEKA) for data acquisition and analysis. For continuous whole cell current recordings oocytes were routinely clamped at a holding potential of −60 mV. Starting from this holding potential voltage-step protocols were performed with consecutive 400-ms step changes with potentials ranging from −100 mV up to 40 mV in 20-mV increments. The average current values reached during the last 100 ms of the voltage steps were used for the current-voltage (I/V) plots. Amiloride-sensitive whole cell currents (ΔIamil) were obtained by washing out amiloride (2 μM) with amiloride-free ND96 and subtracting the whole cell currents measured in the presence of amiloride from the corresponding whole cell currents recorded in the absence of amiloride.

**Surface Labeling of Oocytes**—Experiments were performed essentially as described (32, 34–37) using mouse anti-FLAG M2 monoclonal antibody (Sigma) as primary antibody and peroxidase-conjugated sheep anti-mouse IgG (Chemicon, Boronia Victoria, Australia) as secondary antibody. Individual oocytes were placed in a white U-bottom 96-well plate, and 50 μl of SuperSignal ELISA Femto Maximum Sensitivity Substrate (Pierce) was added to each oocyte. Chemiluminescence was quantified with a Tecan GENios microplate reader (TECAN, Crailsheim, Germany). Results are given in relative light units.

**Cell Culture and Transfection**—FRT cells were seeded on 12-mm diameter, Millipore, North Ryde, NSW Australia) and maintained at 37°C in Dulbecco’s modified Eagle’s medium/F-12 medium (Sigma) containing 5% fetal calf serum (Sigma), 100 units/ml penicillin, and 100 μg/ml streptomycin, as described by others (38, 39). Cells were transfected with siRNA and plasmids containing cDNAs encoding for NDRG2 and α-, β-, and γ-ENaC using Lipofectamine 2000 (Invitrogen) reagent.
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in Dulbecco’s modified Eagle’s medium/F-12 medium without antibiotics 1 day after seeding. After 6 h of incubation medium was replaced by Dulbecco’s modified Eagle’s medium/F-12 medium containing antibiotics, 5% fetal bovine serum, and 10 μM amiloride.

**Ussing Chamber**—Ussing chamber studies were performed 2 days after cell transfection in a continuous perfusion chamber system with a self-built amplifier connected via a MacLab/4 (ADInstruments, Bella Vista, Australia) to an Apple PowerPC (Apple, Cupertino, CA) running Chart (ADInstruments) recording software. Apical and basolateral compartments were continuously perfused at 37 °C with a solution containing (in mM) NaCl 130, CaCl2 1, KCl 4, MgCl2 1, glucose 5, HEPES 10, pH 7.4. Transepithelial resistance (Rₑ) was determined by applying repetitive current pulses (10 μA for 1 s) across the epithelium. Transepithelial voltage (Vₑ) was measured under open circuit conditions, and equivalent short-circuit current (Iₛₛ) was calculated according to Ohm’s law. Conventionally, a lumen negative Vₑ corresponds to a positive Iₛₛ, which may be due to electrogenic cation absorption or electrogenic anion secretion or a combination of both. Amiloride-sensitive Iₛₛ (Iₛₛ−ami) was determined as the difference in Iₛₛ before and after apical amiloride (10 μM) application.

**Western Blot Analysis**—10 oocytes per group were homogenized with a 27-gauge needle in 500 μl of PBS buffer supplemented with protease inhibitor (Complete Mini EDTA-free protease inhibitor mixture tablets, Roche Diagnostics, Mannheim, Germany). Debris was removed by centrifugation at 1,500 × g for 5 min, and 27 μl of the resulting supernatant was loaded, separated by SDS-PAGE (10–12%), and transferred to a polyvinylidene difluoride membrane in a semi-dry blot chamber. Membranes were blocked by incubation in PBS/Triton X-100 (0.05%) with 5% milk powder for 1 h at room temperature, followed by 1-h incubation with a polyclonal rabbit anti-NDRG2 serum (target sequence PGQTPEAAKE, Neosystem, Strasbourg, France) 1:1,000 in PBS/Triton X-100 (0.05%) with 5% milk powder, and finally for 1 h in a secondary antibody (goat anti-rabbit IgG-horseradish peroxidase, Santa Cruz Biotechnology, Santa Cruz, CA) 1:10,000 in PBS/Triton X-100 (0.05%) with 4% milk powder. Detection was performed with the ECL Plus Western blotting detection reagent (Amersham Biosciences). Similarly, the β-subunit of ENaC (anti-β-rENaC antibody, 1:2,000, kindly provided by Bernard C. Rossier) and β-actin (rabbit anti-actin antibody, A2066, Sigma, 1:5,000) were detected on the same blot after stripping. FRT cells were detected on the same blot after stripping. FRT cells were incubated overnight with rabbit polyclonal antibody and further processed as described above.

**Statistical Methods**—Data are presented as mean ± S.E. Statistical significance was assessed by appropriate version of Student’s t test or one-way analysis of variance with Newman-Keuls post test. N indicates the number of different batches of oocytes; n indicates the number of individual oocytes or filters studied.

**RESULTS**

**NDRG2 Stimulates ENaC-mediated Whole Cell Currents in X. laevis Oocytes**—Because NDRG2 is up-regulated by aldosterone and is expressed in nephron segments where ENaC is present, we tested whether co-expression of NDRG2 can affect ENaC activity in X. laevis oocytes. Fig. 1A shows a representative whole cell current trace from an ENaC expressing control oocyte (left) and another trace from an oocyte co-expressing ENaC and NDRG2 (right). Recordings were started in the presence of amiloride in a concentration of 2 μM known to specifically inhibit ENaC. At a holding potential of −60 mV washout of amiloride revealed a sizeable inward current component, which corresponds to the ENaC-mediated sodium inward current. Re-addition of amiloride instantaneously returned the whole cell current toward the initial baseline level. As illustrated by these traces, the amiloride-sensitive whole cell current (ΔIₙₐᵢ₋ₚ) was larger in oocytes co-expressing ENaC and
NDRG2 than in the control oocytes expressing ENaC alone. In similar recordings from oocytes of the same batch $\Delta I_{\text{ami}}$ (at $-60$ mV) averaged 3.5 ± 0.3 μA in ENaC and NDRG2 co-expressing oocytes ($n = 15$) compared with 1.1 ± 0.1 μA in ENaC expressing control oocytes ($n = 15, p < 0.0001$). Average I/V data from this batch of oocytes are summarized in Fig. 1B. The subtracted I/V plots represent the average $\Delta I_{\text{ami}}$ values obtained from voltage step protocols performed in the absence and presence of amiloride as shown in Fig. 1A. From these I/V plots, it is apparent that the average amiloride-sensitive whole cell conductance (estimated between $-80$ and $-100$ mV) was significantly larger in oocytes co-expressing NDRG2 and ENaC (84.3 ± 8.0 μS, $n = 15$) than in control oocytes expressing ENaC alone (27.9 ± 2.9 μS, $n = 15, p < 0.0001$). A similar stimulatory effect of NDRG2 on ENaC currents was observed in 7 of 18 different batches of oocytes tested (Fig. 1C). Importantly, we never observed a significant inhibitory effect of NDRG2. Taken together these findings indicate that co-expression of NDRG2 can stimulate ENaC currents in the oocyte expression system. The reason for the absence of an NDRG2 effect in some batches of oocytes is presently unclear but may be the result of a batch-to-batch variability of expression levels of endogenous Xenopus NDRG2 (see below).

The Stimulatory Effect of NDRG2 on ENaC Is Not Dependent on the Presence of Na$^+$ Feedback Inhibition—It is well known that ENaC-expressing oocytes kept in the presence of high extracellular Na$^+$ will become severely Na$^+$-loaded (27). An increase in intracellular Na$^+$ concentration is known to cause feedback inhibition of ENaC by a complex regulatory pathway involving Nedd4/Nedd4-2 and resulting in channel retrieval from the plasma membrane (40–43). Co-expression of NDRG2 may stimulate ENaC by preventing Na$^+$ feedback inhibition as previously reported for the putative ENaC-regulatory protein N4WP5A (33). To test this possibility we performed co-expression experiments in oocytes that were maintained in the presence of a low extracellular Na$^+$ concentration after cRNA injection. This maneuver is known to lower the intracellular Na$^+$ concentration of ENaC-expressing oocytes and to largely reduce sodium feedback inhibition (33, 36, 41). As expected, the reversal potential was shifted to the right in oocytes incubated in low extracellular Na$^+$ (Fig. 2A) compared with the reversal potential observed in oocytes incubated in high extracellular Na$^+$ (Fig. 1B). This confirms previous findings that the apparent intracellular Na$^+$ concentration can be efficiently lowered in ENaC-expressing oocytes by maintaining the oocytes in a low Na$^+$ bath solution (33, 36, 41). However, as shown in Fig. 2A, the stimulatory effect of NDRG2 on ENaC whole cell currents was fully preserved in oocytes maintained in low extracellular Na$^+$. As in oocytes maintained in high extracellular Na$^+$, a significant stimulatory effect of NDRG2 was not observed in all batches of oocytes maintained in low extracellular Na$^+$ (Fig. 2B). When present, however, the stimulatory effect of NDRG2 in low Na$^+$ oocytes was similar to that observed in high Na$^+$ oocytes. In low Na$^+$ oocytes NDRG2 increased $\Delta I_{\text{ami}}$ to 227 ± 15% ($n = 114$ per group, $N = 8$) of the corresponding value in matched control oocytes expressing ENaC alone. Similarly, in high Na$^+$ oocytes NDRG2 increased $\Delta I_{\text{ami}}$ to 254 ± 13% ($n = 85$ per group, $N = 7$) of its control value. The finding that the NDRG2 effect is not visibly altered by preventing Na$^+$ loading of the oocytes makes it unlikely that modulation of Na$^+$ feedback inhibition contributes to ENaC stimulation by NDRG2.

It is conceivable that the NDRG2 effect depends on the baseline ENaC currents of the oocytes, which can vary considerably from batch to batch. To test this possibility we re-analyzed the data presented in Figs. 1C and 2B. The resulting plots are shown in Fig. 3. As can be seen from these plots, there was no apparent correlation between ENaC baseline currents and the magnitude of the stimulatory effect of NDRG2.

The Time Course of the NDRG2 Effect and Its Dependence on the Injected Amount of cRNA—We routinely studied the effect of co-expressed NDRG2 on ENaC currents 2 days after cRNA injection, because we knew from previous studies that at this point ENaC expression is at a high level without compromising oocyte integrity. In contrast, longer incubation times often result in deterioration of the oocytes. However, in a few experiments oocytes survived long enough so that we could study the
time course of the NDRG2 effect for up to 4 days after cRNA injection. Fig. 4A shows the best of four similar time course experiments in which we also varied the amount of NDRG2 cRNA injected (from 0.08 to 2 ng per oocyte). The amount of injected ENaC cRNA was 0.01 ng/subunit/oocyte and was the same for all experimental groups. In ENaC-expressing control oocytes \( \Delta I_{\text{ami}} \) was larger on day 3 after cRNA injection than on day 2, and even larger on day 4 than on day 3, which probably reflects the fact that, with longer incubation periods, more time is available for ENaC synthesis and channel delivery to the plasma membrane (41). In oocytes co-injected with the usual amount of 2 ng of NDRG2 cRNA, \( \Delta I_{\text{ami}} \) was significantly larger than in control oocytes expressing ENaC alone. In these oocytes a significant stimulatory effect of NDRG2 was already observed 2 days \((p < 0.01)\) after cRNA injection consistent with the findings shown in Figs. 1 and 2. Interestingly, the stimulatory effect of NDRG2 co-expression was even more prominent on day 3 \((p < 0.01)\) and on day 4 \((p < 0.001)\) after cRNA injection. Moreover, on day 4 we also observed a significant stimulatory effect of NDRG2 in those groups of oocytes that were injected with only 0.08 ng \((p < 0.01)\) or 0.4 ng \((p < 0.01)\) of NDRG2 cRNA per oocyte. Our findings clearly demonstrate that the stimulatory effect of NDRG2 is sustained and depends on the amount of NDRG2 cRNA injected. The observation that the NDRG2 effect becomes more prominent with a prolonged incubation time may be one explanation for the frequent lack of a significant stimulatory effect of NDRG2 documented in Figs. 1C and 2B, because these batches of oocytes were only studied on day 2 after cRNA injection.

To confirm the heterologous expression of NDRG2 at the protein level we performed Western blot analysis in parallel with electrophysiological measurements as shown in Fig. 4B. For this experiment cRNA for ENaC (0.01 ng/subunit/oocyte) was injected into oocytes either alone or co-injected with different amounts of cRNA for NDRG2 (0.4, 2, and 10 ng). Two days after cRNA injection \( \Delta I_{\text{ami}} \) was assessed, and Western blot analysis was performed in the same oocytes using an NDRG2-specific antibody that recognizes mammalian NDRG2 but probably not the Xenopus homolog of NDRG2 (see “Discussion”). All three groups of oocytes co-expressing NDRG2 and ENaC showed larger \( \Delta I_{\text{ami}} \) values than oocytes from the control group expressing ENaC alone. Somewhat surprisingly, the stimulatory effect of NDRG2 was largest in the group injected with 2 ng of NDRG2 cRNA rather than in the group injected with the highest amount of NDRG2 cRNA (10 ng). In contrast, the Western blot data showed the expected results. Heterologous NDRG2 protein expression was highest in the group of oocytes injected with the largest amount of NDRG2 cRNA (10 ng per oocyte) with less expression in the group injected with 2 ng and even less in those injected with 0.4 ng per oocyte. Nevertheless, even in the group of oocytes injected with 0.4 ng of NDRG2 cRNA there was a clear NDRG2-specific band of the

NDRG2 expression (see “Discussion”). A specific \( \beta \)-ENaC antibody was used to confirm ENaC protein expression. Detection of \( \beta \)-actin antibody was used as a loading control. Numbers of oocytes per group are indicated as n or are given as numbers in parentheses. Average \( \Delta I_{\text{ami}} \) values of oocytes co-expressing ENaC and NDRG2 were compared with \( \Delta I_{\text{ami}} \) values of the corresponding ENaC control groups \((*, p < 0.05; **, p < 0.01; and ***, p < 0.001)\).
expected size confirming efficient heterologous NDRG2 protein expression. The very faint band seen in ENaC control oocytes is of a slightly larger size than the specific NDRG2 band and is probably not related to NDRG2, because the antibody is unlikely to recognize the Xenopus homolog of NDRG2 (see “Discussion”). In the same blots we confirmed ENaC expression by using a specific antibody against βENaC. Interestingly, ENaC protein expression was lowest in the 10-ng NDRG2 group, which is possibly the result of a nonspecific competition of the cRNAs of ENaC and NDRG2. This finding may explain the reduced stimulatory effect of NDRG2 on ΔI\textsubscript{\text{ami}} in the 10-ng NDRG2 group (Fig. 4B). Taken together, our findings demonstrate a clear dependence of NDRG2 protein expression on the injected amount of NDRG2 cRNA. Moreover, in the presence of constant ENaC protein expression the stimulatory effect of NDRG2 increased with an increase in NDRG2 protein expression from the 0.4-ng to the 2-ng NDRG2 group.

**NDRG2 Enhances the Surface Expression of ENaC**—Two different mechanisms can contribute to an increase in ENaC-mediated Na\textsuperscript{+} whole cell currents. One is an increase in channel open probability and/or single channel conductance. The other is an increase in channel surface expression. The detection of FLAG-tagged ENaC by a chemiluminescence assay makes it possible to distinguish between these mechanisms (34–36). Therefore, we used ENaC constructs with a FLAG tag in the extracellular loop (28) to investigate a possible effect of NDRG2 on ENaC surface expression. In preliminary experiments we noticed that the average ΔI\textsubscript{\text{ami}} was considerably lower in oocytes expressing ENaC with FLAG tags in all three channel subunits compared with ΔI\textsubscript{\text{ami}} in oocytes expressing wild-type ENaC. In contrast, oocytes expressing ENaC channels with a FLAG epitope present only in the β-subunit show similar ΔI\textsubscript{\text{ami}} values as wild-type-expressing oocytes (data not shown). Therefore, we used the latter approach for our co-expression experiments. Normalized data from four different batches of oocytes are summarized in Fig. 5. As shown in Fig. 5A co-expression of NDRG2 had the expected stimulatory effect on ENaC currents consistent with our previous findings (Fig. 1). Importantly, co-expression of NDRG2 also increased ENaC surface expression, which was assessed in parallel in matched oocytes from the same four batches (Fig. 5B). The chemiluminescence assay is a semi-quantitative way of estimating ENaC surface expression. Therefore, to validate our findings we performed additional control experiments using oocytes injected with twice the normal amount of cRNA for NDRG2 (2 ng/oocyte). As an additional control oocytes were injected with twice the normal amount of cRNA for αβ2βENaC (0.1 ng/subunit/oocyte; 2βENaC). Two days after cRNA injection ΔI\textsubscript{\text{ami}} (A) and surface expression (B) were detected in matched oocytes with two-electrode voltage clamp measurements and a chemiluminescence assay, respectively. For the detection of surface expression, the β-subunit of ENaC carried a FLAG-epitope in the extracellular loop. Normalized data obtained in four different batches of oocytes (N = 4) are summarized, and the numbers of individual oocytes are given in parentheses for each column. Non-injected oocytes served as negative controls and showed negligible background luminescence and no ΔI\textsubscript{\text{ami}} (not shown).

**A Small Additive Effect of Sgk1 and NDRG2 Can Be Observed in Some Batches of Oocytes**—The serum- and glucocorticoid-inducible kinase (Sgk1) (12, 44) is known to be induced by aldosterone in the renal cortical collecting duct (14) and to activate ENaC heterologously expressed in X. laevis oocytes (13, 45). Recent in vitro studies suggest that NDRG2 is a potential substrate for Sgk1 (25). To investigate a possible link between the stimulatory effects of Sgk1 and NDRG2 on ENaC we performed an additional set of co-expression experiments, which are summarized in Fig. 6. Our results confirm the well-known stimulatory effect of Sgk1 co-expression on ENaC currents. In initial experiments like the one shown in Fig. 6A we were unable to detect an additive stimulatory effect of NDRG2 and Sgk1 on ΔI\textsubscript{\text{ami}}. However, in these experiments the NDRG2 effect per se was very modest compared with the large stimulatory effect of Sgk1. It may be impossible to resolve an additive NDRG2 effect in the presence of a saturating Sgk1 effect. Therefore, we reduced the amount of injected Sgk1 cRNA to adjust the stimulatory effects of Sgk1 and NDRG2 to similar levels. Moreover, we determined ΔI\textsubscript{\text{ami}} on 3 consecutive days after cRNA injection and incubated the oocytes in low Na\textsuperscript{+} to prevent Na\textsuperscript{+} overload and deterioration of the oocytes. As previously reported, the Sgk1 effect is preserved in oocytes maintained under these conditions (36). In the experiment shown in Fig. 6B we detected a significant stimulatory effect of NDRG2 on ΔI\textsubscript{\text{ami}} on days 1, 2, and 3 after cRNA injection. Similarly, a significant stimulatory effect of Sgk1 on ΔI\textsubscript{\text{ami}} was present on days 2 and 3. Co-expression of NDRG2 and Sgk1 revealed a small but significant additive effect of Sgk1 and NDRG2 on day 2. On days 1 and 3 there was a trend that the largest ΔI\textsubscript{\text{ami}} values were observed in the oocytes co-expressing ENaC, Sgk1, and NDRG2. Our findings suggest the presence of a small additive stimulatory effect of NDRG2 and Sgk1 at least in some batches of oocytes. As expected, in oocytes in which NDRG2 had no effect per se, co-expression of NDRG2 did not enhance the

![Figure 5](http://www.jbc.org/content/jbc/282/38/28269/F5.large.jpg)
stimulatory effect of Sgk1 (Fig. 6C). This seems logical, because we cannot expect an additive effect of NDRG2 and Sgk1 in the absence of an NDRG2 effect.

The Short Isoform of NDRG2 Also Stimulates ENaC—In addition to the 371-amino acid NDRG2 protein used so far in this study, Boulkron et al. (20) also reported a shorter 357-amino acid NDRG2 isoform (NDRG2-sh), which lacks 14 amino acids within the N-terminal portion of the protein (Δ26–39). We wanted to know whether the lack of these 14 amino acids is functionally relevant for the stimulatory effect of NDRG2 on ENaC. To test this, we co-expressed ENaC and NDRG2-sh in oocytes and detected \( I_{\text{ami}} \) 2 days after cRNA injection (Fig. 7). In experiments in which the oocytes were maintained in standard high Na\(^+\) solution after cRNA injection we found a significant stimulation of \( I_{\text{ami}} \) by NDRG2-sh in one of nine batches of oocytes with a non-significant stimulatory trend in additional five batches (Fig. 7A). In experiments in which the oocytes were maintained in low Na\(^+\) solution we detected a significant stimulatory effect of NDRG2-sh in four of six batches of oocytes studied (Fig. 7B). Taken together, our results indicate that the 14 amino acids missing in the shorter isoform are not essential for mediating the stimulatory effect of NDRG2 on ENaC.

NDRG2 Stimulates \( I_{\text{SC-ami}} \) in FRT Cells—To demonstrate that the observed functional interaction between NDRG2 and ENaC is not a phenomenon limited to the \( X. \text{laevis} \) oocyte expression system, we also used transiently transfected FRT cells as an additional, epithelial expression system. FRT cells do not express endogenous ENaC. However, in FRT cells grown on permeable filters the activity of transfected ENaC can be con-
veniently monitored by measuring the amiloride-sensitive short-circuit current ($I_{SC-ami}$). Furthermore, we used an siRNA approach to inhibit endogenous NDRG2 expression in FRT cells. As shown by Western blot analysis (Fig. 8A) NDRG2 is endogenously expressed in FRT cells, and transfection with NDRG2 efficiently enhances NDRG2 expression at the protein level. In contrast, transfection with siRNA against NDRG2 essentially abolished the expression of endogenous NDRG2 and largely reduced the NDRG2 overexpression in FRT cells transfected with NDRG2. These results demonstrate that the FRT cells are a suitable system to study the effects of NDRG2 overexpression or down-regulation on ENaC function. Fig. 8B shows $I_{SC-ami}$ values measured in one batch of FRT cells that were either transfected with ENaC alone or co-transfected with ENaC and different amounts of NDRG2. In all filters co-transfected with NDRG2 the $I_{SC-ami}$ values were larger than in control filters transfected with ENaC alone. However, a stimulatory effect of NDRG2 was not observed in all batches of FRT cells (Fig. 9A). This is consistent with the variability of the NDRG2 effect observed in the oocyte expression system and may be caused by variations in the endogenous NDRG2 expression levels in different batches of FRT cells. On average, $I_{SC-ami}$ was increased to 175 ± 27% ($n = 11, p < 0.05$) in FRT cells transfected with 0.15 µg of NDRG2 plasmid per filter with a non-significant stimulatory trend in FRT cells transfected with 0.30 µg per filter (Fig. 9B). Importantly, down-regulation of endogenous NDRG2 expression by siRNA against NDRG2 inhibited $I_{SC-ami}$ in a dose-dependent manner (Fig. 8C). Small amounts of siRNA (20 pmol/filter) had no inhibitory effect on $I_{SC-ami}$, but higher amounts reduced (60 pmol/filter) or even abolished (140 pmol/filter) $I_{SC-ami}$. The large inhibitory effect of 140 pmol of siRNA per filter was found in three independent sets of experiments using different batches of FRT cells (Fig. 9B). Co-transfection with siRNA against NDRG2 did not significantly alter the transepithelial resistance ($R_{t}$) of the FRT cell monolayers measured in the presence of amiloride. $R_{t}$ averaged 4.5 ± 0.3 kΩ cm$^2$ in siRNA co-transfected filters versus 3.6 ± 0.5 kΩ cm$^2$ in control filters transfected with ENaC alone ($p = 0.16$). This demonstrates that siRNA transfection does not impair the integrity of the epithelial monolayers.

**DISCUSSION**

In the present study we demonstrated that co-expression of NDRG2 can stimulate ENaC-mediated Na$^+$ currents in two different expression systems: X. laevis oocytes and FRT cells. The stimulatory effect of NDRG2 was somewhat variable and was not observed in all batches of cells tested. We never observed, however, a significant inhibitory effect of NDRG2 co-expression. Therefore, the observed stimulatory effect is unlikely to be a random effect. Moreover, because the stimulatory effect of NDRG2 on ENaC was found to be similar in two completely different expression systems, it is unlikely to be an artifact.

Different levels of endogenous NDRG2 expression are a possible explanation for the variable effect of co-expressed NDRG2 on ENaC. Indeed, in FRT cells we detected endogenous NDRG2 expression. Down-regulation of endogenous NDRG2 by siRNA was associated with a dramatic decrease of ENaC activity in FRT cells. This finding supports our hypothesis that NDRG2 is important for ENaC function. Indeed, it is tempting to speculate that a minimal amount of NDRG2 expression may...
NDRG2 Stimulates ENaC

![Graph](image)

FIGURE 9. The stimulatory effect of NDRG2 on \( I_{\text{SC-ami}} \) is variable but the inhibitory effect of siRNA is robust. A, summary of average \( I_{\text{SC-ami}} \) data obtained in similar experiments as described in Fig. 8 (B and C). Each circle represents the mean \( I_{\text{SC-ami}} \) value measured in one batch of FRT cells (2–3 filters/batch). Lines connect the data point of each experimental group with that of the corresponding ENaC control group. The open circles represent mean \( I_{\text{SC-ami}} \) values from the representative experiments shown in Fig. 8 (B and C). B, to summarize data from similar experiments performed in different batches of FRT cells, the individual \( I_{\text{SC-ami}} \) values were normalized to the mean \( I_{\text{SC-ami}} \) value of the corresponding ENaC control filters. \( N \) indicates the number of batches of FRT cells, numbers in parentheses indicate the number of individual filters.

be necessary to support ENaC activity. A data base search in NCBI Entrez Protein revealed a *X. laevis* homolog of NDRG2 (accession number Q7ZY73). The peptide recognition sequence of the anti-NDRG2 antibody used in our study does not match the *X. laevis* isoform of NDRG2 and therefore will only recognize the heterologously expressed rat NDRG2 in the oocyte system. This is consistent with the absence of a specific NDRG2 Western blot signal in control oocytes expressing ENaC alone. Some batches of oocytes may have expressed sufficient levels of endogenous *Xenopus* NDRG2 to fully stimulate ENaC currents thereby preventing any additional effect of heterologously expressed rat NDRG2. Because NDRG2 is thought to play a role in cell differentiation, endogenous expression levels of the NDRG2 homolog are likely to vary in different batches of oocytes. However, our NDRG2 antibody is not specific for *Xenopus* NDRG2 (see above). Therefore, we cannot control endogenous expression levels of NDRG2 in oocytes.

With a chemiluminescence-based surface expression assay we could demonstrate that the stimulatory effect of NDRG2 on ENaC is most likely caused by an increase in channel surface expression. Additional studies will be needed to investigate whether the increase in channel surface expression is caused by an increase in channel insertion, a decrease of channel retrieval, or a combination of both effects. Moreover, at present it is not yet clear by which mechanism NDRG2 may affect channel trafficking. A previous study reported that NDRG2 is phosphorylated by Sgk1 *in vitro* and *in vivo* and that this phosphorylation primes NDRG2 to be a substrate for glycogen synthase kinase 3 (25). Interestingly, our experiments indicate that the stimulatory effects of co-expressed NDRG2 and Sgk1 on ENaC can be additive at least in some batches of oocytes (Fig. 6B). Because both Sgk1 and NDRG2 seem to enhance ENaC surface expression, their mechanism of action may converge into a common pathway involved in the regulation of ENaC trafficking.

Different isoforms of rat NDRG2 protein are known: a longer isoform with 371 amino acids and a shorter isoform with 357 amino acids (20). The shorter isoform lacks 14 amino acids at the N-terminal part. At present it is not known whether these isoforms have different functions. Our experiments suggest that both isoforms can stimulate ENaC. NDRG2 is homologous to MESK2 (misexpression suppressor of dominant-negative KSR (kinase suppressor of Ras)) in *Drosophila melanogaster* (20, 46). This suggests that it may play a role in Ras signaling, a pathway also thought to be involved in aldosterone action in mammalian cells (47). In this context it is noteworthy that the closest NDRG2 homolog NDRG4 was found to enhance extracellular signal-regulated kinase 1 or 2 (ERK1/2) phosphorylation in PC12 cells overexpressing NDRG4 after nerve growth factor treatment (48). Nerve growth factor on the other hand induces the expression of \( \beta \)- and \( \gamma \)-ENaC in PC12 cells (49). Thus, the mechanism by which NDRG2 affects ENaC function may be indirect and may involve more than one pathway.

In conclusion we have demonstrated for the first time a functional effect of the recently identified aldosterone-induced protein NDRG2 on ENaC activity in two different expression systems. The stimulatory effect of NDRG2 is most likely caused by an increase in channel surface expression and may be additive to the stimulatory effect of Sgk1. The molecular mechanism underlying the functional interaction of NDRG2 and ENaC is likely to be complex and remains to be elucidated. Because NDRG2 and ENaC are co-expressed in the aldosterone-sensitive distal nephron (20) our findings suggest that NDRG2 is a likely candidate to contribute to ENaC stimulation by aldosterone *in vivo*.

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