WNKs (with no lysine (K)), unique serine/threonine protein kinases, have been best studied in the context of cell volume regulation and ion homeostasis. Here we describe a biological link between WNKs and transforming growth factor (TGF) β-Smad signaling. Both WNK1 and WNK4 directly bind to and phosphorylate Smad2. Knockdown of WNK1 in HeLa cells using small interfering RNA reduces Smad2 protein expression; this decrease is at least partially due to down-regulation of Smad2 transcription. In contrast, phosphorylated Smad2 significantly accumulated in the nucleus as a consequence of depletion of WNK1, resulting in Smad-mediated transcriptional responses. In addition, TGFβ-induced target gene transcripts were increased in WNK1 small interfering RNA cells. These findings suggest WNK1 as a dual modulator of TGFβ-Smad signaling pathways.

WNKs (with no lysine (K)) family protein kinases gained initial attention because of their unique active site organization; the invariant catalytic lysine in WNK isoforms is contributed from strand β2 (kinase subdomain I) rather than from the conventional location, strand β3 (kinase subdomain II) (1, 2). This placement of the catalytic lysine is conserved among WNK orthologs across species, underscoring the functional relevance of this novel position and suggesting a modified catalytic geometry.

Importantly, at least two of the four human WNK family members are associated with a hereditary form of hyperkalemic hypertension known as pseudohypoaldosteronism type II (3). Consistent with disease association, a rodent model heterozygous for the Wnk1 gene displayed hypotension, implying the haploinsufficiency and dosage sensitivity of WNK1 in blood pressure control (4). Evidence is accumulating to suggest that WNKs may regulate membrane kinetics and trafficking of channels and transporters on the cell surface in renal as well as extrarenal tissues (reviewed in Refs. 5 and 6). Although WNKs appear to serve as molecular switches in salt homeostasis and cell volume control, the underlying mechanisms are still in question.

Although WNK1 is an attractive potential target for antihypertensive therapy, its distribution is ubiquitous, ranging from nonpolarized endodermally to polarized epithelially derived cell lines and tissues. In addition, WNK1 transcripts can be detected as early as the preimplantation stage (www.ncbi.nlm.nih.gov/Unigene) and mice in which the WNK1 gene had been disrupted did not survive past embryonic day 13 of gestation (4). These observations strongly suggest that WNK1 acts throughout the body and also plays an essential role during animal development.

Biochemical studies clearly indicate that WNK1 undergoes diverse interactions in signaling pathways. For example, WNK1 acts upstream in the ERK53 mitogen-activated protein kinase (MAPK) pathway and can phosphorylate the MAPK kinase kinases MEKK2 and MEKK3 (7). This supports the idea that WNK1 is distantly related to Ste20p and may act as a MAPK kinase kinase kinase. Several studies showed that WNK1 can be phosphorylated by Akt (8–10). WNK1 lies downstream of phosphatidylinositol 3-kinase to activate the serum- and glucocorticoid-induced kinase (SGK1) (10, 11). WNK1 also phosphorylates and activates oxidative stress-responsive 1/Ste20-related, proline- and alanine-rich kinase (OSR1/SPAK) to regulate ion balance in response to osmotic stress (12, 13). The existence of multiple spliced forms of WNK1 adds complexity to its functional regulation, notably in kidney where a kinase-defective form lacking N-terminal residues is enriched (14, 15). Interestingly, partial cDNA clones of WNK1 and WNK2 were isolated from malignant prostate tissues and pancreatic cancer cells, respectively (16, 17). The expression of mouse WNK1 was also dramatically suppressed in a tumor cell line (18). However, the roles of WNKs in tumorigenesis, if any, are unknown.

Signal transduction pathways regulated by transforming growth factor (TGF) β control a diverse array of cellular processes including embryogenesis, proliferation, differentiation, migration, and programmed cell death. Dysregulation of and genetic alterations in ligands and signal transducers within this signaling pathway often cause disease or developmental defects (19). TGFβ superfamily ligands transduce signals by stimulating the formation of heteromeric complexes of type I and type II serine/threonine kinase receptors. The type II receptor acti-

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3 The abbreviations used are: ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; MEKK, MEK kinase; TGF, transforming growth factor; siRNA, small interfering RNA; GST, glutathione S-transferase; R-Smad, receptor-activated Smad; MH, Mad homology; Q-PCR, quantitative reverse transcription-PCR; RNAi, RNA interference; PAI-1, plasminogen activator inhibitor type 1.
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vates the type I receptor by phosphorylating a specific region known as the glycine-serine or GS domain. Activated type I receptors initiate intracellular signaling through activation of specific receptor-activated Smads (R-Smads) and their subsequent binding to the co-mediator Smad to relay signals into the nucleus, where they regulate numerous target genes (20, 21). Signaling from the TGFβ-Smad pathway often plays an anti-proliferative role in many cell types, and in that context the receptors and signal transducers act as tumor suppressors (19). For example, malfunctions in TGFβ receptors have been observed in different types of carcinomas, and a number of mutations in Smad genes have been linked to tumors as well (22).

Signaling specificity and versatility are present in the TGFβ signaling pathway through diverse regulatory mechanisms and cross-connections among Smad-dependent and Smad-independent as well as TGFβ-dependent and TGFβ-independent pathways. Phosphorylation of the C-terminal serine residues (e.g. Ser465 and Ser467 in mouse Smad2) in R-Smads by the type I receptor is the most critical event in TGFβ signaling. However, the efficiency, intensity, and duration of TGFβ and Smad signaling are tightly regulated by various mechanisms, including inhibitory Smads, ubiquitin-mediated degradation, phosphorylation by other protein kinase signaling pathways, subcellular compartmentalization, and internalization (20, 23–25). These observations suggest that dynamic convergence of inputs via individual signaling nodes and concomitant modifications may fine-tune TGFβ-Smad signaling pathways to elicit diverse cellular outcomes.

In this study, we investigated the roles of WNK protein kinases in TGFβ-Smad signaling and found that both WNK1 and WNK4 directly bind to one or more R-Smads. Significantly, depletion of WNK1 using siRNA modulated the amounts and effects of Smad2 in both negative and positive manners. These results reveal that the WNK protein kinase pathway is a novel biological link to the regulation of TGFβ-Smad signaling.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid Screening—Yeast two-hybrid screens were performed with a neonatal mouse brain cDNA library (gift from M. Henkemeyer, University of Texas Southwestern Medical Center) as described before (26). From 10⁶ yeast transformants screened with an inactive form of the WNK1 kinase domain (residues 217–494 K233M) as a bait, eight colonies were recovered whose interactions were further confirmed by redelivering isolated prey plasmids into bait-containing L40 host strains. Smad2 cDNAs were encoded in-frame in two of the isolated positive candidates (designated as KD-11-1 and KD-11-2 (26)). For pairwise interaction tests in yeast, the L40 strain was co-transformed with bait and prey plasmids using Frozen DNA Electroporation. For pairwise interaction tests in yeast, the L40 strain was co-transformed with bait and prey plasmids using Frozen DNA Electroporation. For pairwise interaction tests in yeast, the L40 strain was co-transformed with bait and prey plasmids using Frozen DNA Electroporation. For pairwise interaction tests in yeast, the L40 strain was co-transformed with bait and prey plasmids using Frozen DNA Electroporation. For pairwise interaction tests in yeast, the L40 strain was co-transformed with bait and prey plasmids using Frozen DNA Electroporation. For pairwise interaction tests in yeast, the L40 strain was co-transformed with bait and prey plasmids using Frozen DNA Electroporation. For pairwise interaction tests in yeast, the L40 strain was co-transformed with bait and prey plasmids using Frozen DNA Electroporation. For pairwise interaction tests in yeast, the L40 strain was co-transformed with bait and prey plasmids using Frozen DNA Electroporation. For pairwise interaction tests in yeast, the L40 strain was co-transformed with bait and prey plasmids using Frozen DNA Electroporation. For pairwise interaction tests in yeast, the L40 strain was co-transformed with bait and prey plasmids using Frozen DNA Electroporation. For pairwise interaction tests in yeast, the L40 strain was co-transformed with bait and prey plasmids using Frozen DNA Electroporation.
antibody (Sigma) and 30 μl of protein A-Sepharose CL-4B beads (Amersham Biosciences) for 3 h at 4 °C. The immunoprecipitates were washed four times with 20 mM Tris, pH 7.5, 150 mM NaCl, and 7.5 mM MgCl₂, and associated proteins were analyzed by immunoblotting. For immunoprecipitation and kinase assays, lysates (0.5–1 mg) were incubated with the indicated antibody and protein A-Sepharose beads for 3–4 h. The beads were washed three times with 0.25 M Tris, pH 7.4, 1 M NaCl, 0.1% Triton X-100, and 0.1% sodium deoxycholate and once with 10 mM Hepes, pH 7.6. Kinase assays with beads, purified recombinant kinases, and indicated substrates were performed in 30 μl of kinase buffer (20 mM Hepes, pH 7.6, 10 μM ATP, 10 mM MgCl₂, 10 mM β-glycerophosphate, 1 mM dithiorthietol, and 1 mM benzamidine) containing 10 μCi of [γ-32P]ATP.

**Transcriptional Assays and Real Time Quantitative Reverse Transcription-PCR (Q-PCR)—**To measure the transcriptional response downstream of endogenous Smad2 in WNK1-depleted cells, HeLa cells were transfected with reporter constructs (ARE-Lux and FAST-1, 3TP-Lux, or the WNK1-depleted cells, HeLa cells were transfected with DNA for transfection was adjusted with empty vectors. After using FuGENE 6 (Roche Applied Science). The total amount of MLX microtiter plate luminometer (Dynex Technologies), and the serum for 72 h, the luciferase activities were measured using an luciferase measurements.

Total RNA was extracted from cells using RNA STAT-60 (Tel-Test), and cDNA was prepared with the Superscript™ II RT kit (Invitrogen). Real time Q-PCR measurements of cDNAs from at least three plates/condition were performed using SYBR green dye to measure duplex DNA formation, and all of the reactions were performed in triplicate on an Applied Biosystems Prism 7900HT sequence detection system. The relative amount of mRNA was calculated by the comparative threshold cycle method using cyclophilin as the internal control. Statistical analyses were performed using Student’s t test. The primer sequences for Q-PCR are as follows: human Smad2, 5’-GTGC-AGACCCCAATTTGTAAAT-3’ and 5’-CAGCTGGTGGAAATTTACACA-3’, or 5’-AGAGAGTTGAGAACCGTATTGTTGCAAC-3’, and 5’-ATAGTGTCAAGGGCAAGTTT-3’; human TGFβ1, 5’-TGCTGAGGCTCAAGTTAAAAGT-3’ and 5’-TAGGTATGCAGCCAGAATTG-3’; human PAI-1, 5’-CACAATCTGACGGGCACT-3’ and 5’-CATGGGGCCTGATTCAACT-3’. Statistical analyses were performed using Student’s t test.

**Subcellular Fractionation—**HeLa or COS7L cells transfected with siRNA oligonucleotides or plasmids were treated with sorbitol or TGFβ, washed with phosphate-buffered saline, and harvested in hypotonic lysis buffer (10 mM Hepes, pH 7.6, 1.5 mM MgCl₂, and 10 mM KCl) with protease inhibitors as above. After swelling for 10 min, the cells were passed through a 22-gauge needle ~30 times followed by centrifugation at 1000 × g for 5 min. The supernatant was further clarified by centrifugation at 14,000 rpm for 30 min, yielding a cytosolic fraction. The pellet was resuspended in hypertonic extraction buffer (20 mM Hepes, pH 7.6, 1.5 mM MgCl₂, 0.42 mM NaCl, and 2.5% glycerol) with protease inhibitors and incubated on ice for 1 h. The nuclear extract was obtained by sedimenting the insoluble material at 100,000 × g for 30 min. 3–8 μg of each fraction was used for immunoblotting.

**Immunofluorescence—**To localize proteins, siRNA-transfected HeLa cells were grown on coverslips at 1 × 10⁵ cells/35-mm well and treated with 200 pm TGFβ for 10 min. Coverslips were rinsed three times with phosphate-buffered saline, fixed in 3.7% formaldehyde, permeabilized with 0.2% Triton X-100, and blocked with 1% bovine serum albumin. Primary antibodies were co-incubated for 2 h at 1:250 (polyclonal anti-WNK1 Q526) and for 8 h at 1:200 (monoclonal anti-Smad2/3 antibody). The images were obtained with a 60 × objective on a Zeiss Axiovert 200M inverted microscope equipped with the 860002 version 2 JP4 filter set (Chroma Technology) and a Cooke SensicamQE 12 bit charge-coupled device camera. Data collection and analyses were done with SLIDEBOOK 4.0 software (Intelligent Imaging Innovations). The exposure times for all of the images were kept constant, and the images were normalized to a fixed gray scale.

**Additional Methods—**Phosphorylation sites were identified by the UT Southwestern core facility as described with some modifications (26). For phosphate labeling, WNK1-depleted HeLa cells were incubated with phosphate-free medium containing 0.22 mCi/ml [32P]phosphate (MP Biomedicals) for 3 h and treated with sorbitol for 30 min. The lysates were then subjected to immunoprecipitation with the polyclonal anti-Smad2 antibody. The proteins were resolved by electrophoresis and visualized by autoradiography. For quantifying protein amounts, immunoblotting was performed using ECL plus Western blotting detection reagents (Amersham Biosciences), and the intensities of fluorescent intermediates were measured by scanning membranes with the FLA-5100 Image Scanner (FUJIFILM).

**RESULTS**

**WNK1 and Smad Signaling**

WNK1 and WNK4 Directly Interact with R-Smads—Yeast-based screening of a neonatal mouse brain cDNA library with an inactive form of the WNK1 kinase domain as the bait identified two cDNA clones encoding Smad2 (designated as KD-11-1 and KD-11-2 (26)), a signal transducer in the TGFβ signaling pathway. The Smad2 cDNA inserts contained the intact MH2 domain, a linker region, and approximately one-third of the MH1 domain (Fig. 1A). Smad2 is not known as a common false positive in yeast two-hybrid screening. Smad2 showed reproducible binding to the WNK1 bait after being reintroduced into yeast. The interaction between WNK1 and Smad2 was weaker than that previously described between WNK1 and synaptotagmin 2 (26).
Smad proteins fall into three subfamilies: R-Smads, co-mediator Smads, and inhibitory Smads. R-Smads, Smads 1, 2, 3, 5, and 8, specifically interact with and are phosphorylated by type I TGFβ receptors. Subsequently, activated R-Smads recruit the co-mediator Smad Smad4 to form heteromeric complexes that enter the nucleus to regulate target genes. In contrast, the inhibitory Smads, Smad6 and 7, are negative regulators (28). To verify the interactions among WNKs and Smads, we generated full-length or nearly full-length clones of Smads 1, 2, 3, and 4 as well as truncated forms of Smad2 in a yeast two-hybrid vector. Pairwise interaction tests demonstrated that the WNK1 kinase domain bound to TGFβ/activin receptor-activated Smads, Smad2 and 3, but not to the bone morphogenetic protein receptor-regulated Smad1 or to Smad4, indicating binding specificity. Our mapping results further showed that the MH2 domain is required for WNK1 binding to Smad2 (Fig. 1A).

Interestingly, the kinase domain of WNK4 also bound to Smad2 in a similar manner, and this observation contrasts with previous analysis of synaptotagmin 2, another WNK1 interactor, which does not bind to WNK4 (26). The ability of Smad2 to bind both WNK1 and WNK4 suggests that it is a common target for both WNK1- and WNK4-mediated signaling pathways. To exclude the possibility that Smad2 may promiscuously bind to Ste20p-related protein kinases, we also tested the interaction of Smad2 with the Ste20p family members, TAO2 and OSR1. Neither of these kinases directly associated with Smad2 (data not shown).

We further established the interaction between WNK1 and Smad2 by co-transfection and co-immunoprecipitation. Myc-WNK1 fragments (residues 216–491 and 180–555) were co-expressed with full-length FLAG-Smad2 in COS7L cells. These proteins co-immunoprecipitated using antibody to the FLAG tag for pull down (Fig. 1B). TGFβ treatment had little influence on their binding, suggesting that the interaction is ligand-independent (Fig. 1C). However, we were unable to detect the endogenous association of WNK1 and Smad2.

**WNK1 and WNK4 Phosphorylate Smad2 in Vitro**—Phosphorylation of the C-terminal SXS motif in Smad2 by the type I receptor is a crucial event in the canonical TGFβ-Smad2 signaling pathway. However, other protein kinases clearly cross-talk with TGFβ-Smad signaling by phosphorylating Smads or being phosphorylated by the TGFβ receptor (24). Other protein kinases that phosphorylate Smads include receptor tyrosine kinases, ERK1/2, Ca2+/calmodulin-dependent protein kinase II, protein kinase C, MEKK1, and stress-activated protein kinase/c-Jun N-terminal kinase (29–34).

To determine whether Smad2 is a substrate for WNK1 and WNK4, we performed in vitro kinase assays with full-length and truncated forms of GST-Smad2. All of the Smad2 proteins tested were phosphorylated by recombinant WNK1 and WNK4 kinase domains (Fig. 2A). Consistent with this observation, endogenous WNK1 immunoprecipitated from COS7L cells was able to phosphorylate recombinant Smad2 proteins, whereas there was no phosphorylation by protein extracted with preimmune serum (Fig. 2B and data not shown).

More than one site was phosphorylated in Smad2 by WNKs. Following phosphorylation by WNK1, Smad2 became immunoreactive with the anti-phosphorylated Smad2 (Ser(P)465/Ser(P)467) antibody (Fig. 2C). From the mass spectrometric analyses, three phosphorylation sites (Ser110 from MH1 domain and Ser260 and Ser465 from MH2 domain) were identifi-
FIGURE 2. Phosphorylation of Smad2 by WNKs.

A, recombinant GST-Smad2 full length, ΔMH1, ΔMH2, MH1, and MH2 were phosphorylated with recombinant WNK1 or WNK4 kinase domain in vitro. The autoradiogram is shown at the top, and Coomassie Blue-stained gel is shown at the bottom. B, endogenous WNK1 was immunoprecipitated with anti-WNK1 serum (Q256) and subsequently used in kinase assays with the indicated GST-Smad2 proteins as substrates. The activity of endogenous WNK1 was assessed by autophosphorylation as described previously (1); no activity was associated with the immunoprecipitate using preimmune serum (data not shown). C, Smad2 was incubated under phosphorylating conditions in vitro either alone (left) or with WNK1 (right). The proteins were transferred to membranes and immunoblotted with anti-phospho-Smad2. D, positions of phosphorylation sites in Smad2 found from mass spectrometric analyses are indicated. Phosphorylation in vitro by WNK1 of Smad2 mutants lacking individual phosphorylation sites was compared with wild type Smad2. An autoradiogram is shown. The lanes showing a significant reduction in Smad2 phosphorylation are marked by an asterisk. E, siRNA-transfected HeLa cells were 32P-labeled for 3 h and subsequently untreated or treated with 0.5 M sorbitol for 30 min. Endogenous Smad2 was immunoprecipitated with a polyclonal anti-Smad2 antibody (Zymed Laboratories), and the autoradiogram is shown along with the calculated fold change at the bottom panel. Separate samples were loaded in duplicate. There is a mobility shift and intensity reduction in WNK1 immunoblotting following stimulation with sorbitol (13, 35). Also note that the amount of Smad2 protein is diminished in WNK1 RNAi cells (lanes indicated by asterisks). IP, immunoprecipitation; IB, immunoblot.
FIGURE 3. Reduction of Smad2 signal in WNK1-depleted cells. A, HeLa cells were transfected with control or WNK1 siRNA oligonucleotides for 72 h and treated with TGFβ or vehicle for 30 min before lysis. Each experiment was performed in duplicate. The same amount of lysate protein was loaded in each lane and immunoblotted with the indicated antibodies. The immunoblot of Smad2 detected with a monoclonal anti-Smad2/3 antibody (BD Biosciences) showed a reduced signal in WNK1 siRNA lanes (asterisk). The amount of phospho-Smad2 induced by TGFβ was comparable between control and WNK1 RNAi lanes (double asterisks). B, quantification and comparison of protein levels in WNK1-depleted or control cells. Each condition was performed in triplicate, and the resulting samples were loaded for immunoblotting. Quantification of signal intensities was as described under “Experimental Procedures.” The error bars represent the S.D. for the three samples. The doublet signals for Smad2 and actin were due to the gel electrophoresis conditions. C, quantification of Smad2 and Smad4 transcript amounts in WNK1-depleted or control cells. Material from HeLa cells transfected with control or WNK1 siRNA oligonucleotides was subjected to either immunoblotting (top panels) or Q-PCR analysis (bottom graph). The error bars representing the standard error of the mean are shown, and the asterisk indicates statistical significance (p < 0.009) using a Student’s t test comparing control and WNK1 siRNA-treated samples. The amount of Smad2 mRNA was calculated using cyclophilin as the internal control. D, cells were transfected with siRNA as in C with 10 μM MG132 for up to 8 h to inhibit proteasomal degradation. Smad2 was immunoblotted as in C. E–J, immunofluorescence signals of endogenous WNK1 and Smad2 in siRNA-transfected HeLa cells with or without TGFβ treatment. The cells were incubated with control or WNK1 siRNA oligonucleotides for 72 h and subsequently treated with 200 pm TGFβ or vehicle for 10 min. The subcellular localizations of WNK1 and Smad2 were examined by fluorescence microscopy. The WNK1 signal was substantially reduced in F, indicating successful RNAi knockdown of endogenous WNK1. Endogenous Smad2 was also markedly diminished in WNK1 siRNA cells (H). The TGFβ induced Smad2 nuclear localization in WNK1-depleted cells and control cells (I and J). Scale bar, 20 microns. Ctrl, control.
fied (Fig. 2D, top panel, and data not shown). Phosphorylation on Ser465 is presumed to account for the cross-reactivity of WNK1-phosphorylated Smad2 by the anti-phospho-Smad2 (Ser(P)465/Ser(P)467) antibody. Serine residues are conserved in positions that align with Ser110 and Ser465 in Smad1 and 3 but not at the position comparable with Ser250, which is only found in Smad2. The contribution from individual phosphorylation sites was examined by mutagenesis and subsequent in vitro kinase assays. Phosphorylation of Smad2 S465A was drastically reduced (~40% of wild type), although there is little or no change in S110A and S260A mutants (Fig. 2D, bottom panel). These findings suggest that at least in vitro, Ser465 is a major site of Smad2 phosphorylation by WNK1 and WNK4.

Osmotic stress is the only well defined stimulus for WNK1 (1, 35). To determine whether Smad2 is an in vivo substrate for WNK1 following osmotic stress, endogenous WNK1 in HeLa cells was depleted with sequence-specific siRNAs, the cells were labeled with $^{32}$Porthophosphate, and half were treated with 0.5 M sorbitol. Activation of WNK1 by osmotic stress results in the appearance of multiple WNK1 bands with decreased mobility and reduced band intensity (Fig. 2E, first panel) (35). Endogenous WNK1 was successfully knocked down with any of three different siRNA oligonucleotides. Phosphorylation of endogenous Smad2 was not obviously attenuated by WNK1 depletion (Fig. 2E, last panel). Nevertheless, it remains possible that Smad2 may be a WNK1 substrate. One possibility is that phosphorylation by other kinases under these conditions mask any contribution of WNK1.

**WNK1 Depletion Results in a Decrease in Smad2**—In Fig. 2E, the amount of Smad2 protein was decreased by WNK1 RNAi knockdown (Fig. 2E, bottom panel, marked with the asterisk, and Fig. 3, B and C), whereas the amount of actin remained unchanged. To explore this finding further, we performed WNK1 siRNA transfection in the presence or absence of TGFβ and measured Smad2. Consistently, the amount of Smad2 was reduced by WNK1 silencing independent of TGFβ treatment, whereas the amounts of Smad3, Smad4, actin, and lactate dehydrogenase A showed no obvious change (Fig. 3, A–C). Despite the decreased amount of total Smad2 protein, the immunoreactive phosphorylated Smad2 in untreated and TGFβ-stimulated cells from which WNK1 had been depleted was little different from that in cells transfected with control oligonucleotides (Fig. 3A, bottom panel, double asterisks; see below). Quantification indicated that Smad2 was reduced by 20–50% depending on WNK1 siRNA efficiency and cell culture conditions (Fig. 3B and data not shown).

Although it is well documented that Smads are post-translationally regulated by proteasomal degradation, WNK1 silencing appeared to have little impact on Smad2 protein degradation based on the lack of an effect of the proteasome inhibitor MG132 for up to 8 h (Fig. 3D). Instead, real time Q-PCR showed that the amount of the Smad2 transcript was decreased by 20–30% in WNK1 siRNA-targeted cells (Fig. 3C and data not shown). These data suggest that WNK1 may directly or indirectly increase the transcription of Smad2 mRNA.

The subcellular localizations of WNK1 and Smad2 were observed by fluorescence microscopy in WNK1-depleted or control cells. Both endogenous WNK1 and Smad2 were cytoplasmic in the absence of TGFβ. In WNK1-depleted cells very little WNK1 was observed, confirming its successful knockdown in most cells (Fig. 3, E–G). The intensity of Smad2 immunostaining was clearly decreased in the WNK1-silenced cells (Fig. 3H). In the presence of TGFβ, nuclear Smad2 proteins were similarly enriched in both control and WNK1 knockdown cells, despite the reduction in total Smad2 (Fig. 3, I and J). These findings support the quantitation of the immunoblotting experiments.

**WNK1 Reduction Activates Phosphorylated Smad2 Signaling**—Upon activation by TGFβ, Smad2 was efficiently localized to the nucleus even in WNK1-depleted HeLa cells. This raised the interesting possibility that Smad2 signaling might be enhanced in the absence of WNK1. To test whether WNK1 influences TGFβ-Smad2 signaling, we first used RNAi to down-regulate endogenous WNK1 expression and then treated the cells with TGFβ or sorbitol to stimulate WNK1 kinase activity. The amount of active phosphorylated Smad2 remained comparable even in WNK1-depleted cells, which contained less total Smad2 (Fig. 4A, asterisk and double asterisk, respectively). On the other hand, other molecules including actin, ERK1/2, p38, and Smad3 were unchanged in amount. In addition, WNK1-independent, sorbitol-activated protein kinase signaling pathways, p38 and c-Jun N-terminal kinase MAPKs were not dampened by WNK1 reduction, nor was phosphorylated Smad2 signaling modulated by sorbitol.

To evaluate the unexpected observation that the residual Smad2 was phosphorylated to such an extent, we investigated the effect of WNK1 on TGFβ-Smad2 signaling by subcellular fractionation. Depletion of endogenous WNK1 in the absence of TGFβ resulted in an increase in active phosphorylated Smad2 in the nuclear fraction, despite the fact that there was less total Smad2 protein (Fig. 4, B and C). The addition of exogenous TGFβ induced a similar amount of phosphorylated Smad2 in control and WNK1-depleted cells. This further supports the idea that TGFβ-Smad2 signaling is increased by WNK1 depletion. Consistent with this notion, WNK1-deficient primary mouse embryonic fibroblast cells displayed a greater sensitivity to TGFβ than fibroblasts from wild type ani-
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A

| Control RNAi | WNK1 RNAi |
|--------------|-----------|
| TGFi         | Secolob   |
| TGFi         | Secolob   |

- WNK1
- Actin
- ERK1/2
- p38
- Smad3
- P-ERK1/2
- P-p38
- P-JNK
- Smad2
- P-Smad2

B

| Cytosol | Nucleus |
|---------|---------|
| RNAi    |         |
| Ctrl    | WNK1    |
| Ctrl    | WNK1    |

- Lamin B1
- LDH-A
- Smad2
- P-Smad2
- WNK1

C

| TGFi | RNAi | Cytosol | Nucleus |
|------|------|---------|---------|
| 0    | Ctrl |         |         |
| 10 min | WNK1 |         |         |
| 30 min | WNK1 |         |         |

- Rb
- LDH-A
- Smad2
- P-Smad2
- WNK1
FIGURE 4. Activation of Smad2 signaling in WNK1-depleted cells. A, HeLa cells were transfected in duplicate with control or WNK1 RNAi oligonucleotides for 72 h and then treated with vehicle, 200 pM TGFβ for 30 min, or 0.5 M sorbitol for 45 min. Equal amounts of lysate proteins were resolved by SDS-polyacrylamide gel electrophoresis and analyzed by immunoblotting with the indicated antibodies. B, cytosolic and nuclear fractions were prepared as described under “Experimental Procedures” with the indicated siRNA-transfected HeLa cells in the absence of TGFβ. An increase in active phospho-Smad2 in WNK1-depleted cells is indicated by an asterisk. Lamin B1 and lactate dehydrogenase A were immunoblotted as nuclear and cytosolic markers, respectively. C, HeLa cells treated with control or WNK1 siRNA oligonucleotides were treated with TGFβ or vehicle at different times. Subcellular fractions of siRNA-treated HeLa cells were prepared as above in B, and retinoblastoma was detected by immunoblotting as a nuclear marker. A significant enhancement in active phospho-Smad2 was observed (indicated by an asterisk). Serum was present in the duplicate experiments of A–C. Ctrl, control; Rb, retinoblastoma.

FIGURE 5. Smad2 in WNK1−/− mouse embryo fibroblasts. Fibroblasts from WNK1−/− and wild type embryos were either untreated or treated with TGFβ for 10 or 30 min. The same amounts of lysate proteins were analyzed by immunoblotting with the indicated antibodies. Phospho-Smad2 immunoreactivity following TGFβ treatment was increased in WNK1−/− lysates (asterisk).

DISCUSSION

WNK protein kinases are involved in blood pressure control. Among likely contributing mechanisms is the regulation of membrane dynamics of channels and transporters in renal epithelia and elsewhere in the body. Although WNK4 expression is most abundant in kidney, WNKs are also widely expressed in extrarenal tissues. Indeed, WNK kinases have been implicated in diverse roles throughout early development and in survival and proliferation. First, Wnk1-deficient mice display embryonic lethality (4). Fly WNK (CG7177) was identified from genome-wide RNAi screening as a molecule required for cell survival (42). The same molecule was also found as a negative regulator of the Wingless induction-independent Wnt signaling pathway, which is critical for embryonic development, proliferation, differentiation, and polarity (43). An analysis of somatic mutations of 518 protein kinase genes in breast and lung cancers revealed missense mutations in WNK1 and WNK2 (44, 45). Additionally, WNK1 expression was greatly decreased in a tumor cell line, in which ganglioside synthesis was suppressed by genetic manipulation, indicating a putative role of WNK1 in tumorigenesis (18). Recently, WNK3 was reported to promote cell survival by inhibiting caspase-3, thus allowing cells to evade apoptosis (46). All of these findings sug-
WNK1 and Smad Signaling

In this study, we report for the first time that TGFβ-Smad signaling can be modulated by WNK protein kinases. WNK1 and WNK4 directly interact with Smad2 and other R-Smad(s). Importantly, whereas siRNA-mediated knockdown of WNK1 decreased the transcription of Smad2 leading to a decrease in Smad2 protein, WNK1 depletion enhanced active phosphorylated Smad2 signaling and increased Smad2/3-dependent transcriptional responses. These findings suggest that removal of WNK1 releases an inhibitory constraint on Smad2 that enhances TGFβ signaling. In support of this idea, the resulting phosphorylated Smad2 signaling increased the transcription of certain Smad2/3-mediated target genes including TGFβ itself. Autocrine actions of TGFβ may contribute to enhanced Smad2 signaling despite the decrease in Smad2 expression. Because WNK1 and WNK4 kinase domains both interact with Smad2, it is possible that all four WNK family members may affect Smad2 function. Thus, some of the actions of WNKs on survival and proliferation may result in part through the modulation of Smad signaling. The dual mode of WNK1 action in which WNK1 positively regulates the amount of Smad2 in cells and negatively regulates the nuclear accumulation of phosphorylated Smad2 also probably contributes to the complex feedback regulatory mechanism for TGFβ-Smad2 signaling in a cell context-dependent manner.

In addition to its key effector Smad2, TGFβ activates other signaling cascades, such as MAPK pathways. Other stimuli, such as epidermal growth factor or hepatocyte growth factor, also activate Smads (23). These observations reveal substantial cross-connections among Smad-dependent and -independent pathways. Phosphopeptide analyses of endogenous Smads identified a number of phosphorylated residues in addition to the C-terminal serine residues whose phosphorylation is catalyzed by the TGFβ type I receptor. This finding implies roles for additional protein kinases in Smad signaling (31, 47). Our results suggest that WNK1 must be included among candidate kinases that may impact the function of this pathway by direct phosphorylation of Smads. Based on our in vitro data, WNK1 not only interacts with Smad2 through its MH2 domain but also phosphorylates it on both MH1 and MH2 domains. Phosphorylation of Smad2 residues including Ser110, Ser260, and Ser465 may reflect as yet unknown regulatory mechanisms controlled by WNKs, although the cellular stimuli and in vivo sites for WNK1 phosphorylation of Smad2 remain to be investigated.

Other kinases phosphorylate some or all of these same sites on Smad2. One outcome of these phosphorylations may be inhibition of Smad nuclear localization and consequently

FIGURE 6. Transcriptional responses in WNK1-depleted cells. A, HeLa cells were transfected with the indicated siRNAs, reporter constructs (3TP-Lux, ARE-Lux/FAST-1, or the tumor necrosis factor α promoter-Lux), and β-galactosidase or pRLSV40-Lux for 72 h. Luciferase activity in lysates was measured, and the transfection efficiency was corrected to β-galactosidase activity or normalized to luciferase activity from the control promoter. A WNK1 immunoblot showing the knockdown in the tumor necrosis factor α experiment is shown. The data are presented as means ± S.D. of triplicate experiments. RLU indicates the relative luciferase unit of activity. B, quantification of the amounts of TGFβ1 and PAI-1 transcripts was performed as described in Fig. 3. Ctrl, control.
down-regulation of TGFβ-Smad signaling (30, 31). Perhaps more interesting, at least in vitro WNK primarily phosphorylates one of two key residues in Smad2, Ser^{365}, also phosphorylated by TGFβ type I receptor. Phosphorylation of two serine residues (Ser^{465} and Ser^{467}) within a C-terminal SXS motif by the TGFβ receptor is a critical event for intracellular signaling; these phosphorylations do not occur in an ordered manner (48). How a single phosphorylation affects Smad2 function is unknown.

The promoter region of the human Smad gene contains multiple putative SP1-binding sites, and its expression varies among tissues (49). Transcriptional regulation of Smad2 expression is not otherwise well understood. We found that WNK1 knockdown modestly decreased the amount of Smad2 transcript; this decrease may account for the reduction in Smad2 protein in cells from which WNK1 has been depleted. At least one function of WNK1 is as a MAPK kinase (MAP3K) kinase (7), and therefore WNK1 may influence protein kinase signaling pathways that regulate transcription factors for Smad2 expression (23). It remains possible that WNK1 also regulates the stability and fate of Smad2 post-transcriptionally.

Most striking, the decreased amount of Smad2 in WNK1-silenced cells did not result in a decline in TGFβ-Smad signaling. Rather, with WNK1 depletion, Smad2 signaling was enhanced, and phosphorylated Smad2 was significantly enriched in the nucleus even without the addition of exogenous TGFβ. This increase correlated well with transcriptional activation of the Smad2/3-mediated reporters, 3TP-Lux and ARE-Lux. Enhanced Smad2 signaling ultimately led to up-regulation of TGFβ-induced target genes including TGFβ itself and PAI-1. Smads and activating protein-1 are known to regulate the autoinduction of TGFβ (37, 39). Recently it was shown that reduced c-Jun binding to activating protein-1 sites in the TGFβ gene promoter activates TGFβ-mediated autoinduction (50). In fact, transcriptional cooperation among TGFβ-Smad signaling and other signaling pathways including MAPK pathways are well documented (51, 52).

Selective induction of a subset of target genes may be due to selective inhibitory effects on Smad2 signaling, leaving Smad3 signaling apparently unaffected. Culture in serum may also contribute to the selective effects on certain genes. Serum was depleted. At least one function of WNK1 is as a MAPK kinase (7), and therefore WNK1 may influence WNK1 activity. Certain TGFβ signaling pathways are well documented (51, 52).

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