Neuronal apoptosis inhibitory protein is expressed in developing kidney and is regulated by PAX2

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Dziarmaga, Alison, Pierre-Alain Hueber, Diana Iglesias, Nancy Hache, Aaron Jeffs, Nathalie Gendron, Alex MacKenzie, Michael Eccles, and Paul Goodyer. Neuronal apoptosis inhibitory protein is expressed in developing kidney and is regulated by PAX2. Am J Physiol Renal Physiol 291: F913–F920, 2006. First published May 30, 2006; doi:10.1152/ajprenal.00004.2006.—During fetal kidney development, the extent of ureteric bud (UB) branching will determine final nephron endowment for life. Nephron number varies widely among normal humans and those who are born at the low end of the nephron number spectrum may be at risk for essential hypertension in adulthood. Little is known about how nephron number is set. However, we previously showed that the transcription factor, Pax2, suppresses apoptosis in UB cells during kidney development and optimizes branching morphogenesis. Here, we report that Pax2 directly binds to a specific recognition motif in the human neuronal apoptosis inhibitory protein (NAIP) gene promoter. NAIP is an endogenous inhibitor of apoptosis, inactivating caspase-3 and caspase-7 in neuronal tissues. Pax2 activates NAIP gene transcription (7-fold) in vitro and NAIP transcript level is increased fourfold in HEK293 cells stably transfected with Pax2. We show that Naip is expressed in embryonic day 15 (E15) fetal kidney tissue (RT-PCR) and NAIP protein is demonstrated by immunohistochemistry in E15 mouse kidney collecting ducts and P1 proximal tubules. Naip mRNA is significantly reduced (50%) in heterozygous Pax2 mutant mice. Finally, we show that an antisense Naip1 cDNA transfected into murine collecting duct cells doubles caspase-3/7 activity induced by Baxa. These observations suggest that the powerful effects of Pax2 on renal branching morphogenesis and final nephron number may be mediated by activation of Naip which then suppresses apoptosis in UB cells.

Naip (Birc1); kidney development; renal hypoplasia

Development of the metanephric kidneys begins when the ureteric bud (UB) emerges from the wall of the nephric duct and grows laterally into the adjacent mesenchyme and begins to arborize. Signals from the tip of each UB branch induce formation of individual nephrons. When nephrogenesis finally comes to an end (~1 mo before birth in humans), the number of UB branching events by that time will determine nephron number for life. Although there are multiple genes required for branching morphogenesis, the molecular mechanism by which nephron number is set remains unknown.

When congenital nephron number is severely reduced, renal functional capacity is insufficient for postnatal life and children develop progressive renal failure as they grow. About 40% of pediatric end-stage renal disease is due to some form of congenital hypoplasia (2). Even in the “normal” population, however, nephron number varies widely, ranging from 300,000 to over one million per kidney (7). This was once dismissed as a benign reflection of human diversity but recent evidence suggests that subtle renal hypoplasia may be of considerable clinical importance. Mice with heterozygous Gdnf mutations have a significant decrease (30%) in total nephron number. These mice develop glomerular hypertrophy and hypertension as adults (9). This observation is remarkably similar to the report of Keller et al. (22) who found that patients with essential hypertension have roughly 50% fewer nephrons than age-matched controls.

In 1995, Sanyanusin et al. (42, 43) reported that a rare form of autosomal dominant renal hypoplasia, Renal-Coloboma Syndrome (RCS), is caused by mutations of the developmental transcription factor, Pax2. In this syndrome, renal hypoplasia is associated with defects of the optic nerve (36). Some patients lacking obvious eye findings were initially assigned the diagnosis of “oligomeganephronia,” based on renal biopsies showing an absolute reduction in nephron number associated with striking hypertrophy of glomeruli (40). Subsequently, the patients were also found to have heterozygous Pax2 mutations (40), suggesting that Pax2 is centrally involved in setting nephron number.

Pax2 is one of the nine members of the “paired-box” family of transcription factor genes and is normally expressed in fetal midbrain-hindbrain region, the eye, the ear, and the kidney (11). During kidney development, Pax2 is first expressed in the nephric duct during its caudal descent and then in the branching UB (4, 11). Fetal mice with heterozygous Pax2 mutations exhibit a striking increase in apoptosis of UB cells (36). Pax2 inactivation also enhances apoptosis in mIMCD-3 cells derived from murine collecting duct (46). Targeted expression of a proapoptotic gene (Baxa) to the fetal UB increases apoptosis and reduces the number of branching events by embryonic day 15 (E15.5), mimicking RCS (12). Furthermore, the renal branching deficit in Pax2 mutants can be reversed in vitro and in vivo by the caspase inhibitor z-VAD-fmk (8). Thus Pax2 appears to suppress programmed cell death in the UB lineage during branching morphogenesis of the kidney. This effect is critical to achieve optimal nephron number at birth (12).

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The mechanism by which PAX2 suppresses the pathways of programmed cell death is unknown. We examined expression of apoptosis-related genes in HEK293 cells stably transfected with PAX2. This focused our attention on the neuronal apoptosis inhibitory protein (NAIP) gene, also known as BIRC1, encoding an endogenous caspase inhibitor. Although NAIP was initially discovered during positional cloning of the spinal muscular atrophy gene and NAIP protein was subsequently shown to inhibit caspases-3 and -7 in neural tissue (27), we show that NAIP is also expressed in fetal kidney UB. PAX2 directly binds to a motif in the NAIP promoter and stimulates transcriptional activity in vitro. NAIP transcript levels are decreased in Pax2\textsuperscript{2Neu} mutant mice. Unlike humans who have a single functional NAIP gene, mice have multiple copies. However, we show that the Naip transcript is abundant in fetal kidney and an anti-sense Naip cDNA enhances apoptosis in cultured murine kidney collecting ducts.

**MATERIALS AND METHODS**

**RNA isolation.** Total RNA was isolated from mouse kidneys at fetal stages E15.5 and E18.5, at postnatal day 1, and adult kidney of wild-type and Pax2\textsuperscript{2Neu} mice. Briefly, tissues were microdissected and placed in RNAlater (Ambion, Austin, TX) at 4°C. Each kidney was homogenized in TRIzol (Invitrogen, Carlsbad, CA) for 5 min and then mixed with 0.2 volumes of chloroform and incubated at room temperature for 10 min. Samples were centrifuged at 12,000 rpm for 15 min at 4°C. The aqueous layer was removed and equal volume of 70% ethanol was added. Samples were then processed with the RNeasy kit (Qiagen, Valencia, CA) as per manufacturer’s recommendations and total RNA was eluted in 30 μl of RNase-free water. Total RNA was also isolated from cultured cells with TRIzol (Invitrogen) as above or with phenol/chloroform extraction followed by DNaseI treatment (Promega, Madison, WI).

**Real-time RT-PCR of IAPs.** Human fetal kidney (HEK293) cells were stably cotransfected with a hygromycin resistance vector and a pUHD mannian expression vector containing a CMV-driven full length human PAX2b CDNA or an empty vector control (46). Transfectants were selected in hygromycin and cloned. PAX2 expression was confirmed by Western immunoblotting. RNA samples from cells ± PAX2 were analyzed for the following inhibitors of apoptosis (IAPs): XIAP, NAIP, HIAP1, HIAP2, and Survivin, by real-time RT-PCR (with GAPDH as internal standard) as previously described (30). The comparative CT method was used for relative quantification between HEK293/PAX2(+) and HEK293/PAX2(−) cells (26).

**RT-PCR of total Naip transcripts in mIMCD-3 cells.** Total RNA (200 ng or 1 μg) was reverse transcribed by using a one-step RT-PCR kit (Qiagen) with gene-specific primers as per manufacturer’s recommendations. Mouse Naip primers were designed for a 487-bp fragment of the coding region spanning exons 2–9. The primers contained conserved sequences common to all mouse Naip isoforms (21). Naip forward: 5’-GGGACATCATCACAGTGTACCT-3’; Naip reverse: 5’-TGTGTTGCTCTTGATTGGG-3’; Gapdh forward: 5’-AAGGGCTCATAGACCACAGTC3’; and Gapdh reverse: 5’-CATACTTGCGAGATTCTCAAC-3’. RT-PCR conditions were as follows: a 30-min reverse transcription step at 50°C proceeded by an incubation at 95°C for 15 min. A total of 35 cycles were performed at 94°C for 1 min, 58°C for 1 min, 72°C for 1 min, followed by 72°C for 10 min. Samples were run on a 1% agarose gel. The product sizes for Naip and Gapdh were 487 and 252 bp, respectively.

**Real-time RT-PCR assay of total Naip transcripts in Pax2\textsuperscript{2Neu} (+/−) mutant mice.** cDNA was synthesized from 500 ng of mouse kidney total RNA using random hexamer primers (Promega) and Superscript III (Invitrogen) as per the manufacturer’s recommendation. One microliter of cDNA was used for Sybr Green-based quantitative real-time RT-PCR by using a Prism 7000 Sequence Detection System (ABI). PCR conditions were as follows: 2-min incubation at 95°C, followed by 40 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s. Results were standardized with a housekeeping gene, beta 2 microglobulin (B2m), and the comparative CT method was used for relative quantification (3, 26). Duplicate samples from four different litters of E15.5 fetuses (wild-type, n = 12 and Pax2\textsuperscript{2Neu}, n = 16) and two different litters of postnatal day 1 animals (wild-type, n = 13 and Pax2\textsuperscript{2Neu}, n = 5) were studied. Intron-spanning primers were designed for mouse Naip and B2m as follows: Naip forward primer: 5’-GCCAGGTACCATGAGAGGA-3’; Naip reverse primer: 5’-CACAGGAGAAAACACTGCAC-3’; B2m forward primer: 5’-TGCAGATTTAACGATCGGATG-3’; and B2m reverse primer: 5’-TGCTTGTGATCCATGTT-3’. Amplicons for Naip and B2m were 143 and 75 bp, respectively.

**Real-time RT-PCR of Naip1 transcript in mouse kidney.** For real-time quantitative RT-PCR of Naip1, 100 ng of total RNA for each sample was assayed in triplicate for expression of Naip1 RNA simultaneously with mouse Gapdh mRNA. RT-PCR reactions were performed in the ABI Prism 7700 Sequence Detection System (Perkin Elmer Applied Biosystems, Foster City, CA) with the TaqMan EZ RT-PCR kit (Perkin Elmer Applied Biosystems) and TaqMan Rodent Gapdh control reagent (Perkin Elmer Applied Biosystems). Reactions were performed according to manufacturer’s recommendations. Briefly, 25-μl RT-PCR reactions contained: 1× TaqMan EZ Buffer, 3 mM Mn(OAc), 300 μM deoxy-ATP, -CTP, -GTP, 600 μM deoxyUTP, 100 nM rodent Gapdh primers and probe, 600 nM mouse Naip1 primers, 200 nM mouse Naip1 probe, 0.25 μl AmpErase UNG, and 2.5 U rTth DNA Polymerase. RT-PCR conditions were: 2 min at 50°C, 30 min at 60°C, 5 min at 95°C, followed by 40 cycles of 15 s at 94°C and 1 min at 60°C. Samples were normalized for Gapdh mRNA content and expressed as fold induction. Primers and probes used for Naip1 were: forward primer: 5’-TTCTCTTGGGCGGAAGCTTT-3’; reverse primer: 5’-TGCACTTTTCCCCCTGGA-3’; and probe: 5’-AGCATGCCAGTGTCCACAAAAGT-3’.

**Western immunoblotting.** Stably transfected HEK293 cells (+/− PAX2) were incubated on ice for 15 min in 10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 0.5 mM PMSF, and protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany), NP-40 was then added (0.3%) and the mix was vortexed, incubated on ice for 1 min, and centrifuged at 13,000 rpm for 5 min. The supernatant was discarded and the pellet was resuspended in lysis buffer containing 20 mM HEPES, pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1 mM PMSF, protease inhibitory cocktail (Roche Diagnostics) and glycerol (25%). Following a 1-h incubation on ice, the lysate was centrifuged at 13,000 rpm for 5 min and the supernatant was removed and stored at −70°C until ready to use.

Cytoplasmic protein was extracted from whole postnatal day −1 mouse kidney tissue. Briefly, kidneys were homogenized in 500 μl of RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM PMSF, 1 mM EDTA, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 1% Triton X-100, 1% Na Deoxycholate, 0.1% SDS), incubated on ice for 20 min, centrifuged at 13,500 rpm for 25 min at 4°C, and the supernatant was removed and stored at −70°C until ready to use. Extracts containing 30 μg of protein (Pierce Biotechnology, Rockford, IL) were resolved on a 10% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane (Bio-Rad, Mississauga, Ontario). Blots were probed with a 1:200 dilution of polyclonal anti-NAIP antibody (AbCam) or a 1:250 dilution of polyclonal anti-mPAX2 antibody (Zymed, San Francisco, CA) followed by a 1:1000 dilution of anti-rabbit IgG secondary antibody (Perkin Elmer Life Sciences, Boston, MA), detected with an enhanced chemiluminescence detection system (Amersham, Picataway, NJ) and exposed to X-ray film. The expression of β-actin (Oncogene Research Products, San Diego, CA) to normalize for loading differences.
Immunofluorescent staining. Kidneys from wild-type CD1 animals were microdissected at fetal age day 15.5 and postnatal day 1 and fixed in 4% paraformaldehyde (Sigma, St. Louis, MO) for 4 h at 4°C. Kidneys were rinsed briefly and then kept overnight in cold PBS. Tissues were placed in 15% sucrose/PBS until they sank, followed by 30% sucrose/PBS incubation and then placed in Tissue-Tek OCT (Sakura, Torrance, CA) at room temperature for 2 h before freezing on dry ice. Fourteen-micrometer sections were washed in PBS for 5–10 min, dried at room temperature for 10 min, and fixed in cold acetone for 10 min at room temperature. Sections were incubated in universal blocking horse serum in PBS for 1 h at room temperature, followed by an overnight incubation at 4°C with a 1:25 dilution of a polyclonal IgG rabbit anti-hNAIP antibody (Abcam, Cambridge, MA). Sections were then incubated with a 1:200 dilution of goat IgG anti-rabbit Texas Red (Vector Laboratories, Burlingame, CA) in a 0.1 M sodium bicarbonate, 0.15 M sodium chloride buffer, pH 8.5, for 1 h at room temperature in the dark. For double labeling, sections were also incubated overnight at 4°C with fluorescein-labeled lectins, Dolichos Biflorus Agglutinin (DBA), to identify collecting duct tubules and Lotus Torenginosus Lectin (LTL) to identify proximal tubules (Vector Laboratories), mounted with fluoromount (Sigma), and visualized with a Zeiss microscope. This antibody did not work on paraffin-embedded sections.

Vector construction and assay of promoter activity in vitro. A 780-bp SmaI and an end-filled AgeI fragment containing the 5′ flanking sequence of the human NAIP gene (−780 to +1) was ligated into pGL2-basic upstream of the luciferase reporter using SmaI sites. NIH3T3 cells were cultured in monolayer and transiently transfected with the reporter vector using FuGene (Roche, Laval, QC) in the presence and absence of a full-length human PAX2b cDNA in pcDNA3. Cells at 60–70% confluency were transfected with 0.5 μg of human NAIP-Luciferase, 0.5 μg of human PAX2b cDNA or empty vector control, and 5 ng of renilla luciferase as a standard control. Cells were harvested after 48 h posttransfection and lysed in a 1× passive lysis buffer (Promega) and assayed for firefly and renilla luciferase using the Dual Luciferase Kit (Promega) as per the manufacturer’s recommendations. Transfections were performed (6 replicates) on three separate occasions.

EMS. Initial screening of the 780-bp human NAIP promoter sequence was done by MatInspector software. Putative PAX2 binding sites were identified and 40-bp oligos were synthesized, annealed, and labeled with [α-32P]dCTP via the Klenow fill in reaction. Binding reactions containing buffer and purified human PAX2b protein were incubated on ice for 15 min, after which hot probe was added, and reactions were further incubated on ice for 30 min in a total volume of 20 μl. Samples were resolved at room temperature on a 6% nondenaturing polyacrylamide mini gel in 0.25× TBE at 90 V. Gels were then dried at 80°C for 30 min in a vacuum and exposed to film at room temperature overnight. To confirm specificity of DNA/protein complexes, reactions were also incubated with rabbit anti-PAX2 antibody (Zymed) or with mutated oligonucleotides. Competitive binding assays were performed with cold unlabeled probe at 50× and 100× excess. Full-length human PAX2b cDNA cloned into pcDNA3 was used to synthesize purified protein by a coupled in vitro transcription-translation system (TNT/T7, Promega) as per the manufacturer’s recommendations and run on a 4% SDS-PAGE gel to confirm specificity: NAIP oligo: 5′-TGCCAAGTGTAGTGCAAGCCAAATCATTGCTAATGTTGATGACCCT-3′; NAIP mut: 5′-TGCCAAGTGTAGTGCAAGCCAATAATGTCTAAGTGATGGC 3′.

Naip1 knockdown in vitro in cultured cells. To study the effects of Naip1 knockdown, murine inner medullary collecting duct (mIMCD-3) cells were cultured in DMEM medium supplemented with 10% fetal bovine serum and with 1% penicillin/streptomycin in six-well plates. Cells that were 50–60% confluent were transiently cotransfected with Bax cDNA and mouse Naip1 anti-sense cDNA. The Naip1 anti-sense construct is a 566-bp fragment of the first coding exon (exon 2) of the mouse NAIP gene cloned into the pCIS plasmid (20). One microgram of total DNA (0.5 μg of Bax and 0.5 μg of either Naip anti-sense or empty pCI control) using FuGene (Roche) reagents was then used in a volume of 50 μl and combined with 50 μl of caspase 3/7 substrate with buffer and incubated at room temperature for 1 h in the dark. Samples were assayed for caspase 3/7 activity by measure of firefly luciferase activity according to the manufacturer (Promega). Experiments were repeated on three separate occasions in duplicate.

RESULTS

PAX2 selectively activates endogenous NAIP in HEK293 cells. The HEK293 human embryonic kidney cell line expresses low levels of PAX2 protein. To determine whether PAX2 has an effect on the transcriptional activation of IAP genes, HEK293 cells were stably transfected with a CMV-driven expression vector containing the full-length human PAX2b cDNA. Stably transfected HEK293/PAX2 cells exhibited high levels of PAX2 protein by Western immunoblotting compared with control cells transfected with empty plasmid (Fig. 1A). Total RNA was extracted from the cells and analyzed by real-time RT-PCR for changes in transcript levels of various IAP family members (HAIP1, HAIP2, XIAP, Survivin, and NAIP). Of these, only NAIP showed a significant increase (4-fold ± 0.20 SE) in transcript level compared with control cells transfected with an empty plasmid (P < 0.01; Fig. 1B).
Naip is expressed in developing mouse kidney. Unlike humans who bear only one functional copy of the NAIP gene (51), mice have undergone gene duplication events resulting in six separate but highly homologous Naip genes (13, 52). To determine whether one or more Naip genes is expressed in the developing mouse kidney, we first assessed total Naip transcripts in RNA from E15.5, E18.5, P1 mouse kidney tissues, and mIMCD-3 cells (derived from murine collecting duct) by qualitative RT-PCR using primers for a completely conserved region spanning exons 2–8. 

NAIP protein was identified as a single 140-kDa band by Western immunoblotting, confirming specificity of our antibody (Fig. 2). Earlier time points were not studied. NAIP protein was identified as a single polyclonal antibody raised against human NAIP. Earlier time points were not examined. To demonstrate NAIP protein expression in the developing mouse kidney, we performed Western immunoblotting on whole P1 kidneys and immunofluorescent microscopy on frozen sections of normal E15.5 mouse kidney, using a rabbit polyclonal antibody raised against human NAIP. Earlier time points were not studied. NAIP protein was identified as a single 140-kDa band by Western immunoblotting, confirming specificity of our antibody (Fig. 2B). As a marker for fetal collecting ducts, sections were costained with FITC-labeled DBA. As a marker for proximal tubules, the sections were costained with LTL. NAIP expression, detected with an anti-hNAIP primary antibody and Texas Red-tagged secondary antibody, was strong in all collecting duct structures (Fig. 2, C, D, E). By postnatal day 1, NAIP staining was also evident in proximal tubules (Fig. 2, F, G, H). Subcellular NAIP staining pattern is broader than that of DBA or LTL luminal markers (implying cytoplasmic and/or basolateral localization) but also seems to involve some NAIP protein at the luminal membrane. These expression patterns show some overlap with those of PAX2, which is strongly expressed in both collecting ducts and proximal tubules of fetal kidney (4, 36). Sections stained with secondary antibody alone showed no signal. 

Naip mRNA expression is reduced in fetal kidney of Pax2<sup>Neu</sup> (+/−) mutant mice. Total RNA was isolated from wild-type and heterozygous Pax2<sup>Neu</sup> mutant mouse kidney tissues at E15.5 and postnatal day 1 (P1). Qualitative real-time RT-PCR was used to assess Naip mRNA (normalized to beta 2 microglobulin mRNA) in both fetal (E15.5) and newborn (P1) mouse kidneys. In E15.5 Pax2<sup>Neu</sup> heterozygous mutant kidneys, Naip mRNA was significantly reduced (50%) compared with wild-type controls (P < 0.005). Naip mRNA was similarly decreased in newborn Pax2<sup>Neu</sup> mutants (P < 0.05).

PAX2 activates the NAIP promoter in vitro. Initial screening of the 780-bp 5′ flanking sequence of the human NAIP gene revealed a site homologous to the published core consensus sequence for PAX2 recognition motifs (Fig. 4A). This motif was located 140 bp upstream of the transcriptional start site. To determine whether PAX2 activates the human NAIP promoter, a 780-bp fragment of the human NAIP 5′ flanking sequence was cloned into a luciferase reporter vector (Fig. 4B). NIH3T3 cells were transiently cotransfected with the NAIP promoter reporter and an expression vector containing the full-length PAX2b cDNA. Luciferase activity was increased 6.7-fold (±0.05 SD) in the presence of PAX2b compared with empty vector controls (1.0 ± 0.17 SD; P < 0.001; Fig. 4C).

PAX2 protein binds directly to the NAIP promoter. EMSA were used to demonstrate direct binding of human PAX2 protein to the human NAIP promoter. PAX2b protein was synthesized in vitro; presence of PAX2 protein in the translation mix was confirmed by Western immunoblotting (Fig. 5A). For EMSA, a 40-bp oligonucleotide containing the putative PAX2 binding motif was radiolabeled and incubated with PAX2b protein. A DNA/protein complex was observed that

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Fig. 2. Naip expression in fetal mouse kidney. Naip mRNA was detected in RNA from murine mIMCD-3 cells by RT-PCR (A). Naip mRNA was also detected in fetal (E15.5, E18.5) and newborn (P1) mouse kidney tissues (A). Naip protein is seen as a 140-kDa band in extracts of whole P1 kidney (B). In frozen sections of E15.5 mouse kidney, collecting duct cells stained with FITC-labeled dolichos biflorus agglutinin (DBA; C) also expressed NAIP protein (D). Coexpression of NAIP and DBA in collecting ducts is clearly demonstrated in the merged image (E). Newborn proximal tubular cells staining with lotus tetragonolobus lectin (LTL; F) also expressed NAIP protein (G). The coexpression of NAIP in proximal tubule cells is also demonstrated in a merged image (H).

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disappeared in the presence of PAX2 antibody (Fig. 5B). To confirm the specificity of the putative PAX2 binding motif, we performed competitive assays, incubating the protein and hot probe with 50× and 100× excess cold probe. Incubation with cold probe resulted in the disappearance of the DNA/protein complex (Fig. 5C). Furthermore, we mutated 2 bp in the conserved region of the published consensus sequence (Fig. 5E) and repeated the EMSA. When mutated oligos were incubated with PAX2b protein, a band shift was no longer observed (Fig. 5D).

**Naip1 is expressed in fetal mouse kidney.** In preliminary experiments using gene-specific primers for quantitative RT-PCR, we found that multiple *Naip* isoforms are expressed in mouse kidney (data not shown). However, *Naip1* (the most homologous to human *NAIP*) was a dominant transcript. *Naip1* levels in E15 normal fetal mouse kidney were 7.4-fold higher (1.87 SE) than in adult kidney (1.0 ± 0.138 SE; *P* < 0.0001; Fig. 6).

**Naip1 antisense cDNA enhances apoptosis induced by Baxα in IMCD cells.** In previous studies, we showed that mice expressing a Baxα transgene in renal collecting ducts caused increased apoptosis of those cells, mimicking the effects of Pax2 haploinsufficiency (12). To ascertain whether Naip1 inactivation affects susceptibility of murine collecting duct cells to apoptosis, we transiently transfected mIMCD-3 cells with an expression vector containing the full-length murine *Bax*-α cDNA to initiate the caspase cascade (Fig. 7). Cells were cotransfected with control (empty pCI vector) or a Naip1 antisense cDNA expression vector previously shown to inactivate Naip1 expression in neuronal cells (19). This antisense vector contains a 566-bp sequence complementary to the first exon of *Naip1*.
coding exon of Naip1 (20). mIMCD-3 cells cotransfected with anti-sense Naip1 showed a twofold (192 ± 35% SE) increase in Baxo-induced caspase 3/7 activity (P < 0.05).

**DISCUSSION**

Studies of mice and humans with RCS clearly implicate PAX2 in the regulation of final nephron number (36, 42, 43). During normal branching morphogenesis, UB cells rarely undergo programmed cell death but in mice with heterozygous Pax2 mutations there is a dramatic increase in the fraction of UB cells undergoing apoptosis. This phenomenon was primarily seen in the elongating trunks of the UB rather than at UB tips in the nephrogenic zone (36). Our previous studies show that there is a direct relationship between the susceptibility of UB cells to programmed cell death, the extent of UB branching during development, and congenital nephron number (36).

The effects of PAX2 on programmed cell death have been confirmed in a variety of settings. In cancers where PAX2 is aberrantly overexpressed, PAX2 inactivation with siRNAs was shown to sensitize the cells to apoptosis induced by chemotherapeutic agents (29). PAX2 promotes survival of cultured HEK293 cells overexpressing caspase genes (46) or renal collecting duct cells exposed to high-salt concentrations (6). Increased PAX2 expression has also been observed in renal cystic epithelium (49). Heterozygosity for a null Pax2 mutation increased cyst cell apoptosis and reduced cyst size in the cpk mouse (33).

Although it is now clear that PAX2 suppresses apoptosis during organogenesis, the mechanism by which it promotes cell survival is currently unknown. Here, we show that PAX2 directly binds to specific recognition motifs in the human NAIP gene promoter and specifically activates its transcription. In our studies, addition of polyclonal PAX2 antibody eliminated the complex formed by PAX2b protein and an oligonucleotide containing the putative PAX2 recognition motif. Presumably, this antibody interferes with interactions between the binding domain of PAX2b protein and the recognition motif. Numerous other reports show that specific antibodies do not always cause a supershift but can often eliminate complex formation (15, 37). The sevenfold activation of NAIP transcription by PAX2 demonstrated in vitro appears to be physiologically relevant as total Naip transcript levels were significantly reduced in heterozygous Pax2 mutant mice.

The NAIP gene encodes a member of a family of endogenous caspase inhibitors called IAPs. Eight human IAPs have been identified (24). These show a certain degree of tissue specificity and are frequently overexpressed in cancer cells (45). Specifically, NAIP inhibits caspases-3 and -7 (27) and has been shown to suppress apoptosis in neural tissues (16, 20, 25, 34, 50). NAIP was initially discovered during positional clonalization of the causative gene for spinal muscular atrophy (SMA) (39). It was later confirmed that mutation of the survivor motor neuron (SMN) gene adjacent to NAIP is responsible for SMA (23). However, patients with deletions spanning both genes present with a significantly more severe form of SMA (1, 39).

Although only one functional NAIP has been identified thus far in humans (39, 51), six highly homologous but separate Naip genes have been identified in the mouse (16, 52). Thus knockout of the murine Naip1 homolog did not cause a developmental abnormality of the brain, although neurons exhibited increased susceptibility to kainic acid-induced injury compared with controls (18). Presumably, this reflects the functional redundancy of Naip genes in mice, making them suboptimal models for study of NAIP function in humans. The renal status of humans with SMA and homozygous NAIP deletions is unknown, but our observations predict that increased susceptibility to apoptosis during kidney development should reduce congenital nephron number (36). It is of interest that the Naip1 transcript is 7.4-fold higher in fetal than in adult kidney. This parallels the ontogeny of PAX2 expression during murine kidney development (10, 47). In mouse kidney, the effects of PAX2 may reflect its regulation of the Naip1 gene. However, we cannot rule out similar transcriptional regulation of the other murine Naip homologs.

Developmental pathways regulated by PAX2 in the kidney may also be important for brain development. Homozygous null Pax2 mutant mice are anephric, but also exhibit a massive defect in the fetal midbrain/hindbrain region (14). Pax2 has been shown to activate the glial cell-derived neurotrophic factor (Gdnf) gene in the kidney (5). In developing kidney,
GDNF is essential for outgrowth and arborization of the UB (28, 35, 41) and promotes survival of UB cells (48). GDNF is also expressed in the brain where it is an important trophic signal for neural differentiation and neuronal cell survival (44). However, it is unknown whether PAX2 directly activates Gdnf in brain as it does in kidney. PAX2 activates fibroblast growth factor 8 (Fgf8) at the midbrain-hindbrain boundary, a central organizer during neuronal development which is also expressed in the developing kidney (17, 53).

Similar to the neuroprotective role of NAIP in brain tissues, we show that NAIP also has an anti-apoptotic function in kidney cells. Inner medullary collecting ducts cells were more sensitive to Baxα-induced apoptosis, as measured by caspase 3/7 activity, when cotransfected with a NAIP antisense cDNA. Previous in situ hybridization experiments show that Naip expression colocalizes with Pax2 in the midbrain/hindbrain region, the otic vesicle, and the retina during fetal mouse embryogenesis in fetal kidney, thereby optimizing final nephron number by suppressing programmed cell death during branching morphogenesis in fetal kidney, thereby optimizing final nephron number. Conceivably, NAIP could also play a role in the phenotype of cancer cells and cystic epithelium where PAX2 is inappropriately overexpressed.

Final nephron number ranges widely in humans (0.3 and 1.3 million nephrons/kidney) (19, 32). Recent evidence suggests that subtle renal hypoplasia may have far-reaching clinical implications. Humans born into the lower percentiles of the nephron number spectrum have reduced renal reserve and appear to be at risk for essential hypertension later in life (22). Our previous work suggests that PAX2 is centrally involved in regulating final nephron number by suppressing programmed cell death in the UB lineage. Here, we show that the powerful anti-apoptotic effects of PAX2 may be mediated by activation of the endogenous caspase inhibitor, NAIP. We hypothesize that NAIP activation by PAX2 transiently confers resistance of UB cells to programmed cell death during branching morphogenesis in fetal kidney, thereby optimizing final nephron number. Conceivably, NAIP could also play a role in the phenotype of cancer cells and cystic epithelium where PAX2 is inappropriately overexpressed.

GRANTS

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