Phosphorylation of Pax2 by the c-Jun N-terminal Kinase and Enhanced Pax2-dependent Transcription Activation*

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The Pax family of genes encodes DNA-binding proteins that can both activate and repress transcription of specific target genes during embryonic development. Pax proteins are required for pattern formation and cell differentiation in a broad spectrum of developing tissues. Consistent with its expression in the intermediate mesoderm, the optic cup and stalk, and the otic vesicle, Pax2, a member of the Pax2/5/8 subfamily, is essential for the development of the renal epithelia, the optic cup, and the inner ear. In addition to a DNA binding domain, the Pax2 protein contains a carboxyl-terminal transactivation domain rich in serine, threonine, and tyrosine. In this report, we demonstrate that the Pax2 transactivation domain is phosphorylated by the c-Jun N-terminal kinase, but not the ERK1/2 or p38 MAP kinases and that phosphorylation is coincident with increased transactivation of a Pax2-dependent reporter gene. Activation of JNK by either upstream kinase MEKK1 or DLK or by expression of Wnt signaling proteins significantly enhances Pax2 phosphorylation in cells. In vitro kinase assays using immunoprecipitated JNK or constitutively active, recombinant JNK show phosphorylation of GST-Pax2 fusion proteins. In transfected cells, phosphorylation of Pax2 correlates with increased transactivation of a Pax2-dependent reporter gene, suggesting that serine/threonine phosphorylation of the transactivation domain is important for Pax2 activity. Pax2 can form a complex with the JNK scaffolding protein JIP1, and this interaction is enhanced by activation of the JNK signaling module with the upstream kinase DLK. The data demonstrate that Pax2 is a new target for the JNK signaling module and point to a novel mechanism for mediating Pax-dependent transcription regulation.

The Pax family of genes encodes transcription factors with conserved DNA binding motifs that are required for embryonic development of a variety of tissues in Drosophila, mouse, and humans. In vertebrates, Pax genes can be subdivided into classes based on similar features and embryonic expression patterns (1). The Pax2/5/8 subfamily is characterized by an amino-terminal-paired domain, a conserved octapeptide sequence with similarity to the engrailed homology domain (EH-1), and a partial-paired type homeobox. Pax2/5/8 proteins are transcription regulators that bind DNA via the amino-terminal-paired domain, whereas the carboxyl-terminal region is required for transactivation of target genes (2, 3).

In mouse and man, the Pax2 gene is essential for the development of the kidneys (4), optic cup, and inner ear (5). Pax2 encodes at least two alternatively spliced messages that produce proteins of 392 and 415 amino acids, differing only by a 23 amino acid insertion (6). Genes known to be up-regulated by Pax2 include WT1 (7) and gdnf (8) in the developing kidney and engrailed-2 (9) in the developing hindbrain. In transfected cells, transcription activation requires the Pax2 carboxyl-terminal domain that is rich in serine and threonine residues, which may be potential sites for phosphorylation. In the zebrafish Pax6 protein, the carboxyl-terminal transactivation domain is phosphorylated at multiple serine residues by the mitogen-activated protein kinases (MAPK) p38 MAPK and ERK1/2 to increase the transactivation potential (10). Of the Pax2/5/8 subfamily, only Pax8 has been studied with respect to phosphorylation, though it is not clear which kinases are involved (11). MAP kinase cascades are involved in transmitting signals generated at the cell surface into the cytosol and nucleus and consist of three sequentially acting enzymes: a MAP kinase, an upstream MAP kinase kinase (MEK), and a MEK kinase (MEKK) (12, 13). The extracellular signal-regulated kinase 1/2 (ERK 1/2), the c-Jun N-terminal kinase/stress-activated protein kinases (JNK/SAPK), or the p38 MAP kinases, can translocate to the nucleus and subsequently phosphorylate a variety of transcription factors. MEKK1 phosphorylates MKK4/MKK7 to activate JNK but can also activate ERK and p38 MAPK in transfected cells. This report addresses the phosphorylation state of the Pax2 protein and its ability to activate transcription. We show that the carboxyl-terminal activation domain is phosphorylated at serine and threonine residues and that Pax2 phosphorylation is coincident with the enhanced ability to transactivate a reporter gene. Pax2 is a substrate for the c-Jun N-terminal kinase (JNK) but, unlike Pax6, is not phosphorylated by ERK or p38 MAPK. Activation of JNK by either the upstream kinases MEKK1 or DLK or by expression of Wnt signaling proteins increases Pax2 phosphorylation and enhances the Pax2 transactivation potential. The data point to an important role for JNK in modifying the Pax2 transactivation domain and stimulating Pax2-dependent gene expression.

MATERIALS AND METHODS

DNA Constructs—The CMV-Pax-2bHA and carboxyl-terminal deletions and the PRS4-CAT reported plasmid, containing five tandem

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1 The abbreviations used are: MAPK, mitogen-activated protein kinase; JNK/SAPK, c-Jun N-terminal kinase/stress-activated protein kinase; MEK, MAPK kinase; MEKK, MEK kinase; CAT, chloramphenicol acetyltransferase; HA, hemagglutinin.
Pax2 Phosphorylation by JNK

copies of the pax2 binding site PRS4 cloned upstream of the herpes simplex virus thymidine kinase promoter, were as described (3). The CMV-MEK1 wild-type, CMV-MEK1 K432M, HA-DLK, Myc-Erk, FLAG-JNK, Myc-p38 MAPK, were provided by L. Holzmann. The Myc-JIP1 was a gift of B. Margolis. The Wt4 (gift of A. McMahon), Wt11, and Wt1b (IMAGE Consortium) coding sequences were cloned into CMV-CB6 and sequenced for verification. The Pax2-GST fusion protein contained amino acids 197–415 and was purified by affinity to glutathione-agarose. GST-Elk (Cell Signaling Technology, cat. no. 9184S) and GST-ATF-2 (Cell Signaling Technology, cat. no. 9224S) were obtained commercially. GST-c-Jun (1–223) was described (14). Constitutively active, recombinant JNK was purchased from Upstate Biotechnology (Lake Placid, NY).

Antibodies—The antibodies used in this paper were: Pax-2 (6) 1:3000; FLAG (55 monoclonal antibody, Sigma, 1:2000); HA (16B12, BAbCO, 1:2000); Myc (9E10, BAbCO, 1:2000); phospho-JNK (Thr-183/Tyr-185, Cell Signaling Technology, 1:2000); phospho-Erk1/2 (Thr-202/Tyr-204, Cell Signaling Technology, 1:2000); phospho-p38 MAPK (Thr-180/Tyr-182, Cell Signaling Technology, 1:2000); phospho-c-Jun (Ser-63, Cell Signaling Technology, 1:2000); JNK (Cell Signaling Technology, 1:2000); β-tubulin (TUB 2.1, Sigma, 1:10,000).

Western Blot—Western blots were performed with equal amounts of protein obtained by lysis of transiently transfected cells in PK lysis buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 1.5 mM MgCl2, 1 mM EGTA, 1 mM Na3VO4, 0.1% Triton X-100, 10% glycerol, and a mixture of protease inhibitors (Roche Molecular Biochemicals). The reactions were terminated by the addition of 12 μl of 6X SDS loading buffer, 10 mM EGTA and were subjected to SDS-PAGE followed by transfer to nitrocellulose. The complexes were incubated for 30 min at 30 °C in 50 μl of kinase buffer containing 20 μM ATP, 5 μM of [γ-32P]orthophosphate, 2 μg of GST-Pax2 (197–415), and 2 μg of either GST-Elk-1 (ERK ass, Cell Signaling), GST-c-Jun (JNK assay) or GST-ATF-2 (p38 MAPK assay, Cell Signaling). The reactions were terminated by the addition of 12 μl of 6X SDS loading buffer, 10 mM EGTA and were subjected to SDS-PAGE followed by transfer to nitrocellulose. The amount of kinase that was immunoprecipitated was assessed by re-immunoblotting of the nitrocellulose (Fig. 1). The reactions were performed essentially as described (15). Percent acetylation was determined by scintillation counting, and a value of 1.0 was arbitrarily assigned to control parental plasmids. Each experiment was repeated three times and the results presented as averages ± 1 S.D. from the mean.

RESULTS

Pax2 Is a Substrate for the c-Jun N-terminal Kinase—The activation domain of Pax2 contains multiple serine, threonine, and tyrosine residues that could serve as potential phosphorylation sites. To determine the status of Pax2 phosphorylation, cells were transfected with epitope-tagged Pax2 and labeled with [32P]orthophosphate. Lysates were immunoprecipitated with anti-HA (lanes 1 and 2) or anti-FLAG (lanes 3 and 4) and analyzed on SDS-PAGE gels. Note major phosphoprotein migrating at ~48 kDa and slightly higher molecular mass species at about 50 kDa (arrows). A, amino acid analysis of [32P]-labeled Pax2b immunoprecipitated with anti-HA (1) or anti-FLAG (2). Markers for phosphoserine, threonine, and tyrosine were traced from the TLC plates (dotted lines).

Fig. 1. Phosphoamino acid analysis of Pax2. A, cells were transfected with vector only (lanes 1 and 4) or with HA-tagged Pax2b (lane 2) or FLAG-tagged Pax2b (lane 3) and metabolically labeled with [32P]orthophosphate. Lysates were immunoprecipitated with anti-HA (lanes 1 and 2) or anti-FLAG (lanes 3 and 4) and analyzed on SDS-PAGE gels. Note major phosphoprotein migrating at ~48 kDa and slightly higher molecular mass species at about 50 kDa (arrows). B, amino acid analysis of [32P]-labeled Pax2b immunoprecipitated with anti-HA (1) or anti-FLAG (2). Markers for phosphoserine, threonine, and tyrosine were traced from the TLC plates (dotted lines).
The kinases were immunoprecipitated and used for immunocomplex kinase assays with GST-Pax2 and control substrates. Transfected MEKK1 can activate all three pathways in 293 cells, as demonstrated by increased phosphorylation of ERK, JNK, and p38 MAPK expression plasmids, with (+) or without (−) 0.1 μg of MEKK1. A, fraction of the total lysates were analyzed by SDS-PAGE for activation of MAPK by antibodies against P-ERK1/2, P-JNK, and P-p38 as indicated. Equal loading of lysates was assessed by anti-β-tubulin. Note activation of all three transfected MAPK in the presence of MEKK1. B, the remaining lysates were immunoprecipitated with anti-Myc or anti-FLAG antibodies for in vitro kinase assays. The kinase reactions contained a mixture of two substrates. GST-Pax2 was included in all reactions, whereas the controls contained either GST-Elk, GST-c-Jun, and GST-ATF2 for the ERK, JNK, and p38 assays respectively. Note that GST-Pax2 is phosphorylated by JNK only. C, in vitro kinase reaction using recombinant active JNK and equal amounts of GST-Pax2 or GST-c-Jun as substrates. D, in vitro kinase reactions using GST alone or GST-Pax2 fusion proteins and recombinant JNK. The reactions were done with excess cold ATP and immunoblotted with anti-Pax2 antibodies. Note the slower migrating GST-Pax2 species in the JNK-treated reaction (arrow). Because the original antigen was a Pax2-GST fusion protein, the slower migrating GST-Pax2 species in the JNK-treated reaction is a substrate as GST-c-Jun (Fig. 2B). The ability of activated JNK to phosphorylate Pax2 was confirmed using a recombinant, constitutively active form of JNK1 (Fig. 2C). The in vitro kinase assay using recombinant JNK and the GST fusion proteins as substrates indicated that GST-Pax2 was as efficient a substrate as GST-c-Jun (Fig. 2C). In vitro phosphorylation of GST-Pax2 was also done with an excess of cold ATP, and the proteins were detected by Western blotting (Fig. 2D). Using anti-Pax2 antibodies, a slower migrating form of the GST-Pax2 fusion protein is observed upon phosphorylation with recombinant JNK. Thus, the antibodies can distinguish between the unphosphorylated and phosphorylated forms of the GST-Pax2 fusion protein.

The phosphorylation of Pax2 was also examined directly in cells co-transfected with MEKK1. To map the domain of phosphorylation more precisely, carboxyl-terminal deletion constructs of Pax2b were utilized (Fig. 3). Specific antibodies recognize the 1325-amino acid Pax2b protein, which migrates at ~48 kDa. Upon co-transfection with MEKK1, a slower migrating Pax2b species was observed at ~50 kDa in SDS-PAGE gels (Fig. 3B). Preincubation of the lysates with either calf intestinal phosphatase or potato acid phosphatase eliminates this slower migrating Pax2b form, indicating that the shift in mobility is due to phosphorylation, similar to what was observed in the in vitro assay (Fig. 2D). The MEKK1-dependent, phosphorylated Pax2b form is seen with carboxyl-terminal deletion constructs 1–373, 1–333, but not with deletions that contain only amino acids 1–279. Thus, Pax2b phosphorylation is primarily within the carboxyl-terminal transactivation domain spanning amino acids 279–415. However, phosphorylation at additional sites between amino acids 1–279 that might not affect the mobility of Pax2 in SDS-PAGE gels cannot be ruled out. Strikingly, this region contains 19 serine and 11 threonine residues, making definitive assignment of the phosphorylation sites difficult.

Activation of Pax2 Phosphorylation by Wnt Signals—During kidney and neural development, Wnt signaling is known to mediate inductive interactions. While the canonical Wnt signaling pathway activates the TCF/LEF family of transcription factors by inhibition of the GSK3 kinase and translocation of β-catenin to the nucleus, alternate Wnt signaling pathways can work through the activation of JNK (16, 17). Particularly during the specification of planar cell polarity (18), activation of JNK is known to require Disheveled and is uncoupled from the TCF/LEF pathway (19–21). Given the relationship between Wnt signaling, kidney development, and JNK activation, we examined the ability of Wnt proteins to activate JNK and stimulate Pax2 phosphorylation. Cells were co-transfected with expression plasmids for Wnt4, Wnt11, and Wnt17a and examined for JNK activation and Pax2 phosphorylation (Fig. 4A). Analysis of total lysates from 293 cells co-transfected with Wnt expression vectors and Pax2b indicated co-incident phosphorylation of Pax2, as evidenced by the slower migrating Pax2b isoform (Fig. 4A). Activation of the Wnt pathway results in the slower migration of the disheveled protein, through phosphorylation at multiple sites, as reported previously (22, 23). Cells transfected with each of the three Wnt constructs also exhibited co-incident activation of JNK (Fig. 4A). The phosphorylation of Pax2 was confirmed by in vivo labeling with

![Fig. 2. Pax2 is a substrate for JNK. 293 cells were transfected with either 0.5 μg of Myc-ERK, FLAG-JNK, or Myc-p38 MAPK expression plasmids, with (+) or without (−) 0.1 μg of MEKK1. A, fraction of the total lysates were analyzed by SDS-PAGE for activation of MAPK by antibodies against P-ERK1/2, P-JNK, and P-p38 as indicated. Equal loading of lysates was assessed by anti-β-tubulin. Note activation of all three transfected MAPK in the presence of MEKK1. B, the remaining lysates were immunoprecipitated with anti-Myc or anti-FLAG antibodies for in vitro kinase assays. The kinase reactions contained a mixture of two substrates. GST-Pax2 was included in all reactions, whereas the controls contained either GST-Elk, GST-c-Jun, and GST-ATF2 for the ERK, JNK, and p38 assays respectively. Note that GST-Pax2 is phosphorylated by JNK only. C, in vitro kinase reaction using recombinant active JNK and equal amounts of GST-Pax2 or GST-c-Jun as substrates. D, in vitro kinase reactions using GST alone or GST-Pax2 fusion proteins and recombinant JNK. The reactions were done with excess cold ATP and immunoblotted with anti-Pax2 antibodies. Note the slower migrating GST-Pax2 species in the JNK-treated reaction (arrow). Because the original antigen was a Pax2-GST fusion protein, the slower migrating GST-Pax2 species in the JNK-treated reaction is a substrate as GST-c-Jun (Fig. 2B). The ability of activated JNK to phosphorylate Pax2 was confirmed using a recombinant, constitutively active form of JNK1 (Fig. 2C). The in vitro kinase assay using recombinant JNK and the GST fusion proteins as substrates indicated that GST-Pax2 was as efficient a substrate as GST-c-Jun (Fig. 2C). In vitro phosphorylation of GST-Pax2 was also done with an excess of cold ATP, and the proteins were detected by Western blotting (Fig. 2D). Using anti-Pax2 antibodies, a slower migrating form of the GST-Pax2 fusion protein is observed upon phosphorylation with recombinant JNK. Thus, the antibodies can distinguish between the unphosphorylated and phosphorylated forms of the GST-Pax2 fusion protein.

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![Fig. 3. Phosphorylation of the Pax2 transactivation domain. A, schematic of carboxyl-terminal deletions used for 293 cell transfections is shown. The DNA binding-paired domain (DBD), the octapeptide (O), the partial homeodomain (PHD) and the serine, threonine, tyrosine-rich transactivation domain (STY) are shown. B, Western blot analyses of cell lysates from transfected 293 cells. Pax2 constructs were transfected as indicated, with or without 50 ng MEKK1. A fraction of each lysate was treated with either calf intestinal phosphatase (CIP) or potato acid phosphatase (PAP) as indicated. Note slower migrating Pax2b species is specific for MEKK1-transfected cells and disappears upon phosphatase treatment.
FIG. 4. Wnt signaling activates JNK and phosphorylates Pax2. A, Pax2 (125 ng) was co-transfected with the indicated Wnt expression plasmids (µg) and lysates were analyzed by Western blotting with anti-Pax2 antibodies. Note slower migrating Pax2b species (arrow) is prevalent upon Wnt transfection. Controls include the Pax2 expression plasmid only (P) and a plasmid vector only (C). Lysates were also probed with anti-P-JNK, anti-P-ERK, anti-P-p38, and anti-Dvl. Note activation of JNK and a slower migrating form of Dvl1 (arrow). Equal loading was assessed with anti-β-tubulin. B, metabolic labeling of Pax2. Cells were transfected with Pax2bHA and 500 ng Wnt11 or 50 ng MEKK1 as indicated and labeled for 4 h with [32P]orthophosphate. Pax2 was immunoprecipitated and analyzed by SDS-PAGE. The top panel is an autoradiograph of the HA immunoprecipitate. The bottom panel is an immunoblot with Pax2 antibodies to show levels of protein. Note that both MEKK1 and Wnt-11 increase Pax2 phosphorylation. Control is plasmid vector only without the Pax2 coding region.

[32P]orthophosphate (Fig. 4B). MEKK1 co-transfected cells showed high levels of phospho-Pax2, whereas Wnt11 co-transfected cells showed less phospho-Pax2 but significantly more than cells expressing Pax2 alone. The data indicate that activation of JNK by either Wnt proteins or MEKK1 results in increased phosphorylation of Pax2.

Pax2 Interacts with the JIP1/JNK Signaling Module—Activation of the JNK pathway can also occur with the mixed lineage kinases (MLK or DLK) in association with the scaffolding proteins of the JIP family (14, 24, 25). To determine whether Pax2 interacts directly with this JNK signaling module, cells were co-transfected with Pax2, JIP1, DLK, and JNK (Fig. 5). Expression of DLK alone slightly increased the amount of phospho-Pax2 present in total lysates. At low doses of DLK, Pax2 phosphorylation was further enhanced by co-expression with JIP1 or JNK. Immunoprecipitation of JIP1 also pulled down Pax2 and JNK, indicating that at least some Pax2 was associated with the JNK signaling module. Strikingly, Pax2 could be co-immunoprecipitated with JIP1 in the absence of DLK, but the amount of JIP1-associated Pax2 increased significantly upon activation of the JNK module with DLK. More phosphorylated Pax2 was observed by expression of DLK, together with exogenous JNK, and this resulted in even more JIP1-associated Pax2. In the absence of exogenous JIP1, co-transfection of DLK and JNK produced even more phosphorylated Pax2. At first this may seem counterintuitive, but transfection of exogenous JIP1 provides more scaffolding such that individual proteins may not all assemble on the same scaffold and thus effectively titrate out some of the activities (14). The data suggest that DLK-dependent phosphorylation of Pax2 by JNK is mediated by a direct association with the JNK signaling module, perhaps through interactions with JIP1.

Transactivation of Pax2 Reporter Genes—To assess the effects of Pax2 phosphorylation on the transactivation potential, we utilized a reporter plasmid containing five copies of the PRS4 Pax2 binding sequence upstream of a minimal promoter and the chloramphenicol acetyltransferase (CAT) gene. Using a fixed amount of Pax2b plasmid (125 ng) and increasing amounts of MEKK1, Pax2-dependent reporter activation increased dramatically with as little as 50 ng of MEKK1 expression plasmid (Fig. 6A). Pax2 transfection can stimulate the reporter plasmid activity by 8–10-fold. This increased to more than 50-fold by co-transfection of 200 ng of MEKK1 expression plasmid. In the absence of Pax2, MEKK1 had no effect on the expression of the reporter. Furthermore, a kinase-deficient MEKK1 construct showed only a slight increase at the highest doses. Pax2-dependent transactivation was also enhanced in the presence of Wnt expression plasmids (Fig. 6B). Expression of Wnt4, Wnt11, and Wnt7a all generated a 2–3-fold increase in Pax2-dependent activation of the reporter gene. Whereas Wnt gene expression alone had no effect on CAT activity in the absence of Pax2 (data not shown). As reported previously (3), deletion of the transactivation domain reduced Pax2-dependent activation (Fig. 6C). A carboxyl-terminal deletion of amino acids 373–415 still showed a robust response to MEKK1 although the basal level of Pax2-dependent activation was down about 2-fold. However, a carboxyl-terminal deletion of amino acids 333–415 exhibited a further reduction in basal activation and a loss of responsiveness to MEKK1. Western blotting indicated that there was some degree of Pax2 (1–333) phosphorylation in response to MEKK1 (Fig. 3B). However, it is unlikely that these remaining phosphorylation sites are necessary for the increased activation potential in response to MEKK1. These data suggest that the critical phosphorylated residues in Pax2 that mediate the MEKK1-induced transactivation activation are located between amino acids 333 and 415. Similarly, activation of JNK via DLK and JIP increased the transactivation potential of Pax2 (Fig. 6D). DLK and Pax2 alone increased...
In particular, phosphorylation of Ser-63 and Ser-73 of c-Jun results in increased activity of the AP-1 transcription factor, which consists of a c-Fos/c-Jun heterodimer (13). Indeed, the sequence around Ser-63 of c-Jun (LLTSP) is very similar to a conserved serine at position 393 of the Pax2 protein (LLSSP). However, phosphorylation of Pax2 may occur at multiple sites. Deletion of the serine/threonine-rich, carboxyl-terminal transactivation domain significantly reduces phosphorylation such that a shift in Pax2b mobility, upon JNK activation, is no longer observed. The phosphorylated residues that mediate the JNK-dependent increase in transactivation reside between amino acids 333 and 415 of the activation domain. Within this region there are 11 serine and 7 threonine residues, of which 6 and 1 respectively are absolutely conserved within the Pax2/5/8 family. We have begun making point mutations of these conserved residues. However, preliminary data indicate that multiple serine substitution must be made before the MEKK1-dependent increase in transactivation is abrogated (data not shown).

By definition, the transactivation domain is required for maximal activation potential of the Pax2 reporter gene, PRS4-CAT, as defined previously in the absence of exogenous JNK activators. A basal level of phospho-Pax2 is present that most probably accounts for the 8–10-fold transactivation potential observed without JNK activation. This basal level accounts for the metabolically labeled phospho-Pax2 observed without JNK activation and the small amount of high molecular weight Pax2b form observed upon long exposures of Western blots. Basal levels of activated JNK and phospho-c-Jun are inevitably observed in proliferating cells stimulated with serum. Because there are presently no practical JNK inhibitors, we were unable to test whether JNK activation was absolutely required for Pax2-mediated transactivation. However, several lines of evidence indicate that JNK is the kinase responsible for Pax2 phosphorylation, which correlates with increased transactivation. Of the major MAP kinases, only JNK is able to phosphorylate Pax2 in the immunolinked kinase assay. Furthermore, recombinant constitutively active JNK phosphorylates Pax2 in vitro as efficiently as it phosphorylates c-Jun. Increased JNK activity leads to increased Pax2 phosphorylation and increased Pax2-dependent transactivation, regardless of whether JNK is activated by MEKK1, Wnt signaling, or DLK. Pax2 can associate with the JNK signaling module as demonstrated by the co-immunoprecipitation of Pax2 with JIP1. Strikingly, activation of the signaling module by the DLK results in increased JIP1-associated Pax2. A specific inhibitor of MEK1 (PD98059), which suppresses phosphorylation of the ERK1/2 MAPK, does not significantly reduce Pax2 phosphorylation nor does it reduce Pax2-dependent transactivation (data not shown). While JNK is likely to be the kinase that mediates phosphorylation of Pax2, the effect on Pax2 activity is less clear. It remains to be determined whether Pax2 phosphorylation by JNK is merely co-incidental with enhanced activation, whether it is a prerequisite for further modification by other kinases, or whether there is indeed a causative link to enhanced Pax2 activation potential.

In the developing kidney and central nervous system, Wnt signaling and Pax genes play critical roles in establishing patterns and specifying cell types. During kidney development, Pax2-expressing cells proliferate, aggregate around the tips of the ureteric bud, and undergo conversion to an epithelial phenotype (6, 26). This response to inductive signals is Pax2-dependent (4, 27) and also requires Wnt4 signaling (28). That Wnt4 can activate JNK and stimulate Pax2-dependent gene

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**DISCUSSION**

In this report, we demonstrate that Pax2, a member of the Pax family of transcription regulators, is phosphorylated by JNK. Also known as SAPK, JNK phosphorylates a variety of nuclear transcription factors, including c-Jun, Elk1, and ATF2.

![Transactivation potential of Pax2](image-url)

**FIG. 6. Transactivation potential of Pax2.** A, MEKK1 increases the transactivation of a Pax2b reporter gene. Cells were transfected with Pax2 alone or with increasing amounts of MEKK1, and with the reporter plasmid PRS4-CAT. The control sample has reporter only and the MEKK1 sample contains the reporter and MEKK1. B, Wnt signaling increases Pax2-dependent transactivation. Cells were transfected with Pax2b, PRS4-CAT, and increasing amounts of Wnt expression plasmids. Wnt expression alone has no effect on the levels of PRS4-CAT (data not shown). C, deletion analysis within the Pax2 transactivation domain. Cells were transfected with full-length Pax2(1–415) or with deletion constructs containing the indicated amino acids and with (+) or without (−) MEKK1. Note that Pax2b(1–333) shows no increase in activity caused by MEKK1. D, activation of Pax2b-dependent reporter gene by the DLK/JIP1 signaling module. Note that co-transfection of JIP1 (100 ng) and DLK (100 ng) increases Pax2b-dependent transactivation 3–4-fold. DLK or JIP1 alone have no effect on reporter gene expression. For all panels, the mean values of three independent transfections for CAT activity were shown relative to control expression plasmid, which was assigned a value of 1.0.

**FIG. 6A.** MEKK1 increases the transactivation of a Pax2b reporter gene. Cells were transfected with Pax2 alone or with increasing amounts of MEKK1, and with the reporter plasmid PRS4-CAT. The control sample has reporter only and the MEKK1 sample contains the reporter and MEKK1. B, Wnt signaling increases Pax2-dependent transactivation. Cells were transfected with Pax2b, PRS4-CAT, and increasing amounts of Wnt expression plasmids. Wnt expression alone has no effect on the levels of PRS4-CAT (data not shown). C, deletion analysis within the Pax2 transactivation domain. Cells were transfected with full-length Pax2(1–415) or with deletion constructs containing the indicated amino acids and with (+) or without (−) MEKK1. Note that Pax2b(1–333) shows no increase in activity caused by MEKK1. D, activation of Pax2b-dependent reporter gene by the DLK/JIP1 signaling module. Note that co-transfection of JIP1 (100 ng) and DLK (100 ng) increases Pax2b-dependent transactivation 3–4-fold. DLK or JIP1 alone have no effect on reporter gene expression. For all panels, the mean values of three independent transfections for CAT activity were shown relative to control expression plasmid, which was assigned a value of 1.0.
activation suggests that phosphorylation of Pax2 is an essential component of the induction response. Thus, Pax2 may be an immediate downstream target for inductive signals that can translate these signals by initiating the epithelial-specific genetic program.

Pax2 and the related genes Pax5 and Pax8 are also active during neural development, particularly in the mid-brain/hindbrain junction of the central nervous system (29–31). Mice carrying targeted mutations for both JNK1 and JNK2 show regional defects in the developing hindbrain, including decreased apoptosis prior to neural tube closure in and around the Pax2/5/8 expression domain (32). Thus, modulation of Pax activity by JNK may also be required for the regulation of neuronal cell number through selective activation of apoptosis. In any event, more direct studies in normal and mutant mice are warranted to more precisely determine the phosphorylation state of Pax proteins during specific stages of development.

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