Ptf1a Directly Controls Expression of Immunoglobulin Superfamily Molecules Nephrin and Neph3 in the Developing Central Nervous System*§

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Ptfa, a basic helix-loop-helix transcription factor, plays an indispensable role for cell fate specification of subsets of neurons in the developing central nervous system. However, downstream molecules induced by Ptfa during neural development have not been well characterized. In the present study, we identified immunoglobulin superfamily molecules, Nephrin and Neph3, as direct downstream targets of Ptfa. First, the expression domains of Nephrin and Neph3 closely resembled those of Ptfa in the developing retina, hypothalamus, cerebellum, hindbrain, and spinal cord. Second, Ptfa bound directly to a PTF-binding motif in the 5′-flanking region of Nephrin and Neph3 genes. Third, Ptfa activated transcription driven by the 5′-flanking region of these genes. Finally, the expression of Nephrin and Neph3 was lost in Ptfa-null mice, whereas ectopic expression of Nephrin and Neph3 was induced by forced expression of Ptfa. We provided further evidence that Nephrin and Neph3 could interact homophilically and heterophilically, suggesting that Nephrin and Neph3 might regulate certain developmental aspects of Ptfa-positive neurons as homo- or heterooligomers.

Ptfa, which encodes a basic helix-loop-helix transcription factor, was first identified as a cell fate determinant of the pancreas (1, 2). Mutations in the human and mouse Ptfa result not only in the malformation of the pancreas but also in cerebellar agenesis, indicating its involvement in neuronal development (3, 4). Recent studies demonstrated that Ptfa is expressed in neuronal progenitors in the retina, cerebellum, hindbrain, and spinal cord, most of which are fated to be subsets of GABA (γ-aminobutyric acid)-ergic and glycineric inhibitory neurons (4–10). In the Ptfa-null cerebellum, Ptfa-positive progenitors, which are normally fated to inhibitory neurons, trans-fate to excitatory granule cells (8). Cell fate changes in Ptfa-null mice were also reported in the retina, hindbrain, and spinal cord (5–7, 9). Conversely, ectopic expression of Ptfa in neural progenitors of the dorsal telencephalon, which are normally fated to glutamatergic excitatory neurons, confers inhibitory GABA characteristics to these neurons (4). These loss- and gain-of-function experiments suggest that Ptfa acts as a cell fate switch in the central nervous system. However, how Ptfa operates to fulfill such a critical role is not known because of a lack of understanding of its downstream targets.

In the present study, we searched for direct downstream targets of Ptfa, which is likely to be responsible for cell fate specification of Ptfa neurons. We identified Nephrin and Neph3 as candidates of Ptfa target molecules. Nephrin and Neph3 are transmembrane proteins of the immunoglobulin superfamily. Nephrin was originally isolated as a gene responsible for the congenital nephrotic syndrome of the Finnish type (11). Neph3 belongs to Nephr family, which is composed of Nep1, Nep2, and Neph3 (also called as Kirrel, Kirrel3/mKirre, and Kirrel2/Filtrin, respectively), and Nep family members are structurally related to Nephrin (12). Nephrin and Nep family members can interact homophilically, and in some cases heterophilically, which in turn triggers cell adhesion or signal transduction (13–19). Recent reports showed expression of Nephrin and Nep family molecules in the developing central nervous system (17, 18, 20–22). Although their function in vertebrate neural development is still unclarified, their invertebrate homologs appear to play indispensable roles in synapse formation or axon guidance during development (23, 24).

Here, we provide evidence that Ptfa directly controls the expression of Nephrin and Neph3. Moreover, Nephrin and Neph3 interacted homophilically and heterophilically. These findings suggest that Nephrin and Neph3, via their interaction, play important roles in Ptfa-positive neuronal progenitors.

EXPERIMENTAL PROCEDURES

Vectors—pCAG, pCAG-EGFP, pCAG-mCherry, pCAG-IRESEGFP, and pEF-RL vectors were described previously.
(25–29). Full-length NephrinB cDNA was cloned from mouse E13.5 cerebellum, and its sequence was deposited into DDBJ with accession number AB513652. For construction of expression vectors of NephrinA (87–3857 of NM_019459), NephrinB (1–3729 of AB513652), and Nephr3 (130–2232 of BC052773), cDNAs were subcloned into pCAG vector. A HA or FLAG sequence was inserted between 200 and 201 of NephrinA (NM_019459), between 72 and 73 of NephrinB (AB513652), and between 189 and 190 of Nephr3 (BC052773). For construction of expression vectors of Ptf1a (199–1173 of NM_018809), E47 (87–2033 of AF352579), RBPF1 (217–1716 of NM_009035), N-Twist (66–572 of NM_033522), Math1 (196–1251 of NM_007500), and Neurogenin1 (232–966 of NM_010896), cDNAs were subcloned into pCAG or pCAG-ires-EGFP. A HA, FLAG, or Myc tag was added in their N termini by PCR. pGL2-basic vector was obtained commercially (Promega). The 5′-flanking region of Nephrin and Nephr3 genes was amplified by PCR from genomic DNA of ICR mice and subcloned into pGL2-basic. The following primers were used for PCR amplification: primer 1 (5′-ATG CAA GCT TTG TGT GTA CCC CAA GAT C-3′), primer 2 (5′-GAC CAA GCT TTG TGT GTA CCC CAA GAT C-3′), and primer 3 (5′-GAC CAA GCT TTG TGT GTA CCC CAA GAT C-3′), and primer 3 (5′-GAC CAA GCT TTG TGT GTA CCC CAA GAT C-3′). For amplification of the 1.6-kb sequence upstream of the Nephr3 start codon (see Fig. 4A), primers 1 and 2 were used as forward and reverse primers, respectively. For the 1.6-kb sequence upstream of the NephrinB start codon or 3.3 kb upstream of the NephrinA start codon (see Fig. 4B), primers 2 and 1, or primers 2 and 3 were used as forward and reverse primer, respectively. The E-box and the TC-box mutations were introduced by PCR-based site-directed mutagenesis.

Antibodies—Anti-Ptf1a antibody was described previously (9, 30). Anti-FLAG (M2, Sigma), anti-Myc (9E10, Santa Cruz Biotechnology), anti-HA (3F10, Roche Applied Science), alkaline phosphatase (AP)-conjugated anti-mouse IgG (Bio-Rad), AP-conjugated anti-rat IgG (Jackson Laboratories), AP-conjugated anti-digoxigenin (DIG, Roche Applied Science), peroxidase-conjugated anti-DIG (Roche Applied Science), anti-POX (Molecular Probes), and biotin-conjugated anti-rabbit IgG (Jackson Laboratories) antibodies were obtained commercially.

In Situ Hybridization and Immunohistochemistry—The Ptf1a<sup>−/−</sup> mouse line was described previously (1). In situ hybridization was performed using DIG-labeled RNA probes of Nephrin (2733–3764 of NM_019459) and Nephr3 (1037–2129 of BC052773). For fluorescence detection, samples were incubated with a peroxidase-conjugated anti-DIG antibody, biotinylated-tyramide (PerkinElmer Life Sciences), and Alexa Fluor 594-streptavidin (Molecular Probes). To couple with immunostaining, sections were first hybridized with DIG-RNA probes and immunostained with an anti-Ptf1a (30) or an anti-FGP antibody.

In Utero Electroporation—In utero electroporation into the cerebral cortex of E14.5 mouse embryos was described previously (26). Expression vectors were introduced into E14.5 CR mice, and pregnant mice were killed 1 or 2 days later for further analyses.

Chromatin Immunoprecipitation Assay—Cerebellum from E11.5 CR mice was dissected out, and chromatin immunoprecipitation assay was performed as described previously (31). Endogenous Ptf1a was immunoprecipitated with an anti-Ptf1a antibody (9). The amount of co-precipitated DNA fragments was analyzed by quantitative PCR using a LightCycler (Roche Applied Science). Primers used in quantitative PCR were as follows: PTF1 motif forward (5′-GCC AGG AGT TCA GAT TTA GGT G-3′) and reverse (5′-AGG ATG GGA AAA CCA ACA GAC-3′); 1.5 kb 5′ of the PTF1 forward (5′-TCC TCT TCA CCC ACT CAC AG-3′) and reverse (5′-AGT TCA CAG TGG GTC CAA GC-3′); 1.5 kb 3′ of the PTF1 forward (5′-TAC ACA CAC TCG GGA TGC TG-3′) and reverse (5′-CCA AAC AGG GCT GTA ATG GAC-3′).

Electrophoretic Mobility Shift Assay (EMSA)—EMSA was performed following the manufacturer’s protocol in the LightShift Chemiluminescent EMSA kit (Pierce). Nuclear extracts of HA-Ptf1a−, FLAG-E47−, or Myc-RBPF1-transfected COS7 cells were prepared as described previously (32). Forward and 3′-biotinylated reverse oligonucleotides were purchased commercially (GeneDesign). The sequence of oligonucleotides is shown in Fig. 3B. Nuclear extracts were incubated with 200 fmol of annealed biotinylated oligonucleotides. For the supershift assay, the nuclear extracts were preincubated with anti-HA, anti-FLAG, or anti-Myc antibody. Biotinylated oligonucleotides were detected by streptavidin-AP (Jackson Laboratories) and an AP substrate, CDP star (Roche Applied Science), and chemiluminescence was analyzed by RAS3000 mini (Fuji film).

Luciferase Reporter Assay—Luciferase reporter assay was performed following the manufacturer’s protocol in the Dual Luciferase Reporter Assay system (Promega). pGL2-basic vector constructs and pEF-RL with or without pCAG-Ptf1a were transfected into COS7 cells by FuGENE 6 (Roche). 48 h later, the activity of the firefly and Renilla luciferases was analyzed by Luminometer (Promega).

Affinity Probe in Situ Assay—An affinity probe in situ assay using Nephr3-AP was performed as described previously (18). Binding of Nephr3-AP was detected by AP activity using nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate as substrates. To measure the binding affinity of Nephr3-AP to COS7 cells transfected with HA-Neph3 or HA-Nephina expression vector, cells were incubated with an AP substrate, p-nitrophenyl phosphate, for 2 h. Increase of A<sub>405</sub> in each sample relative to that in mock-transfected samples was analyzed.

Immunoprecipitation and Western Blotting—COS7 cells were transfected with expression vectors by FuGENE 6 or a standard calcium phosphate method. Immunoprecipitation and Western blotting were performed as described previously (13).

RESULTS

Nephrin and Nephr3 Genes Have a PTF1-binding Motif in Their 5′-Flanking Region—Ptf1a is a component of a trimeric transcription factor, PTF1, which interacts with the PTF1-binding motif, comprising an E-box (CAG/CCTG) and a TC-box (TTTCCC) separated by one or two helical turns (33–35). In an attempt to identify novel downstream targets of Ptf1a, we took advantage of a data base, DBTSS, to screen for genes containing PTF1 motif in their 5′-flanking regions. As a result, we found a PTF1 motif located in the shared 5′-flanking region of Nephrin and Nephr3 genes on mouse chromosome 7 (Fig. 1).
The Nephrin gene has two isoforms, NephrinA and NephrinB, resulting from utilizing alternative first exons, 1A and 1B. Thus, the PTF1 motif is located 700 bases upstream of the first exon of Nephrin, and 70 bases and 1.8 kb upstream of exon 1B and exon 1A of Nephrin, respectively.

Nephrin and Nephr3 Are Downstream Targets of Ptf1a—If Nephrin and Nephr3 are downstream targets of Ptf1a, they would be expressed in Ptf1a-positive domains. To examine this possibility, we first analyzed the expression of Nephrin and Nephr3 in the developing central nervous system. Nephrin and Nephr3 were expressed in overlapping regions where Ptf1a is expressed, in the cerebellum and hindbrain at E12.5, in the spinal cord at E10.5, and in the retina and hypothalamus at E13.5 (Fig. 2, supplemental Fig. 1, and data not shown). Many Ptf1a-positive cells appeared to co-express Nephrin and Nephr3 (Fig. 2). Moreover, Nephrin and Nephr3 were co-expressed in the same cells, which was confirmed by double labeling of Nephrin and Nephr3 (data not shown). We further examined the expression of two isoforms of Nephrin in the developing cerebellum by quantitative PCR. Consistent with the previous report (36), comparable amounts of endogenous NephrinA and B were expressed in the developing cerebellum, whereas adult kidney predominantly expressed NephrinA (supplemental Fig. 2). These results raise the possibility that Ptf1a may control the expression of Nephrin and Nephr3.

Ptf1a Binds to the Promoter Region of Nephrin and Nephr3 Genes That Contains PTF1 Motif—Because Nephrin and Nephr3 genes contain the PTF1 motif in their 5′-flanking region, it is highly possible that Ptf1a controls the transcription of these genes by direct binding to the PTF1 motif. We examined this possibility by chromatin immunoprecipitation assay to test whether Ptf1a in the developing cerebellum associates with the 5′-flanking region of Nephrin and Nephr3 genes in vivo. Anti-Ptf1a antibody immunoprecipitated DNA fragments centered on the PTF1 motif, but not 1.5 kb upstream or downstream of the motif (Fig. 3A). This interaction could also be detected in Ptf1a-overexpressed cerebral cortex (supplemental Fig. 3). We further examined whether the interaction could be reconstituted in vitro. Beres et al. (37) reported that PTF1 complex in the pancreas comprised Ptf1a, a class A basic helix-loop-helix protein, such as E47 or HEB, and RBPL. In the developing central nervous system, RBPL instead of RBPL is expressed and interacts with a PTF1-binding motif in vitro (37, 38). Thus, we performed EMSA using oligonucleotides containing the PTF1 motif and a combination of Ptf1a, E47, and RBPL (Fig. 3B). RBPL or an E47 dimer could bind the PTF motif alone. However, addition of all three proteins resulted in a new shifted band, associated with a heterotrimeric complex composed of Ptf1a/E47/RBPL. The presence of Ptf1a/E47/RBPL trimer in the
shifted band was confirmed by the fact that incubation with an antibody for each component all led to a supershift of the band (Fig. 3C). Mutations in E-box abolished Ptf1a binding, whereas the mutated TC-box had no effect in its binding, suggesting that E-box is necessary for the binding of Ptf1a to the PTF1 motif (Fig. 3B). Taken together, these results demonstrated in vivo and in vitro that Ptf1a binds to the promoter region of Nephrin and Neph3 genes and this binding is dependent on the PTF1 motif.

Ptf1a Activates Transcription from the Nephrin and Neph3 Promoter—We next tested whether Ptf1a binding to the promoter region of Nephrin and Neph3 leads to trans-activation of downstream genes by using a luciferase reporter system. Expression of Ptf1a in COS7 cells strongly activated a luciferase reporter driven by the 5'-flanking region of Nephrin and Neph3 containing the PTF1 motif in both directions (Fig. 4, A and B). Note that transcriptional activation by Ptf1a could take place from both promoter fragments upstream of exon 1A and exon 1B of the Nephrin gene, albeit weaker in the former. Mutations in the E-box or TC-box, particularly the E-box, notably reduced the level of Ptf1a-dependent transactivation, suggesting that activation of the promoter depends on the successful binding of Ptf1a to the PTF1 motif. Some other members of basic helix-loop-helix, such as Math1, Neurogenin1, or N-Twist, did not enhance Ptf1a transactivity, and Ptf1a did not activate c-fos promoter (Fig. 4, C and D, and data not shown). Furthermore, co-expression of Ptf1a and E47 caused synergistic enhancement of Neph3 promoter activation (supplemental Fig. 4). To confirm the dependence of Neph3 promoter activation on Ptf1a in vivo, we performed a luciferase assay in the developing cerebellum and cerebral cortex, which are Ptf1a-positive and Ptf1a-negative areas, respectively. As shown in supplemental Fig. 4, Neph3 promoter was highly activated in the developing cerebellum compared with the cerebral cortex, indicating a correlation of the presence of Ptf1a and high activation of the Neph3 promoter. These results suggest that Ptf1a activates transcription from Nephrin and Neph3 promoter.

Ptf1a Is Essential for Expression of Nephrin and Neph3 in Vivo—Ptf1a interacted with the PTF1 motif, thereby inducing trans-activation of the Nephrin/Neph3 promoter, raising the possibility that Ptf1a controls the expression of Nephrin and Neph3 in vivo.
To assess this possibility, we first examined the expression of Nephrin and Neph3 in Ptf1a-null mice (Fig. 5, A–L, and supplemental Fig. 1). The expression of Nephrin and Neph3 was lost in the developing cerebellum, hindbrain, spinal cord, reti-na, and hypothalamus of the homozygotes. We next performed gain-of-function experiments by ectopically expressing Ptf1a in the developing cerebral cortex, where Ptf1a, Nephrin, and Neph3 are not endogenously expressed (data not shown). We found that ectopic Ptf1a triggered a notable induction of Nephrin and Neph3 expression (Fig. 5, M–T), but not the other Neph family members Neph1 and Neph2 (data not shown). Because Ptf1a activated transcription from exon 1A and 1B of the Nephrin gene (Fig. 4B), we further examined whether both Nephrin isoforms are induced by ectopic expression of Ptf1a in the cerebral cortex. As expected, we found induction of NephrinA and B by Ptf1a (supplemental Fig. 2). These results together suggest that Ptf1a controls the expression of two isoforms of Nephrin and Neph3 in the developing central nervous system.

Interaction between Nephrin and Neph3—So far we have shown that Ptf1a simultaneously activates the transcription of Nephrin and Neph3 in subsets of neural precursors. This raises the possibility that Nephrin and Neph3 may interact and in turn control certain aspects of the fate of these cells. Indeed, heterophilic interactions between Nephrin, and Neph1 or Neph2 have been previously demonstrated (13–16). However, a Nephrin-Neph3 interaction has not been reported. We therefore investigated any potential interaction between Nephrin and Neph3 by co-immunoprecipitation experiments in COS7 cells. As shown in Fig. 6A, FLAG-NephrinA was co-precipitated with co-expressed HA-Neph3 or HA-NephrinA, but not with HA-NephrinB. To examine whether NephrinA and Neph3 interact in cis on the same cell, or in trans on different cells, we performed a similar experiment using a mixture of cells transfected with each construct separately (Fig. 6B). However, NephrinA was co-precipitated with neither Neph3 nor itself in this condition. These results imply that NephrinA interacts with Neph3 as well as itself in cis. To investigate whether NephrinA-Neph3 may interact in trans, we performed an affinity probe in situ assay using Neph3-AP fusion protein (Fig. 6, C and D). Consistent with a previous report (18), Neph3-AP bound to Neph3-expressing cells, but not to NephrinA-expressing cells.

It has been shown in invertebrate that trans-interactions between Nephrin and Neph family mediate cell-cell adhesion (23, 24). We thus examined whether NephrinA and Neph3 could mediate cell adhesion. As expected, only Neph3-expressing cells formed cell aggregates, but no aggregation was
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![Diagram of brain sections showing expression of Nephrin and Neph3]

**FIGURE 5.** Nephrin and Neph3 expression in Ptf1a-null mice and Ptf1a-misexpressed cerebral cortex. A–L, expression of Nephrin and Neph3 in Ptf1a-null mice. Expression of Neph3 (A, B, F, J, and I) and Nephrin (C, D, G, H, K, and L) were analyzed by in situ hybridization on coronal sections of E13.5 cerebral cortex (A–D), E13.5 dorsal hindbrain (E–H), and E10.5 spinal cord (I–L). Ptf1a<sup>cre/+</sup> mice (A, C, E, G, I, and K) and Ptf1a<sup>cre/cre</sup> mice (B, D, F, H, J, and L) were used. Dorsal is upward. Scale bar, 100 μm. M–T, expression of Nephrin and Neph3 in Ptf1a-overexpressed cerebral cortex at E16.5. Expression of Nephrin (N and P) and Nephrin (R and T) were analyzed by in situ hybridization on coronal sections of IRES-EGFP-transfected (M, N, O, and R) or Ptf1a-IRES-EGFP-transfected (O, P, S, and T) cerebral cortex. Expression of EGFP on the same sections was analyzed by immunostaining by anti-EGFP (M, O, Q, and S). Insets in P and T are high magnification views of merged images outlined in O, P, S, and T. Dorsal is upward. Scale bar, 100 μm.

detected between Neph3- and NephrinA-expressing cells and between NephrinA-expressing cells (supplemental Fig. 5). These data, taken together, showed that NephrinA-Neph3 interactions take place in cis but not in trans, whereas Nephr3 could interact homophilically in trans, thereby mediating cell adhesion.

**DISCUSSION**

In the present study, we identified Nephrin and Neph3 as direct downstream targets of Ptf1a in the developing central nervous system. Our finding provides an important clue to unravel as yet unclarified molecular mechanisms underlying cell fate specification of Ptf1a-neurons. Henke et al. (39) recently reported Neurog2, itself a basic helix-loop-helix transcription factor, as a first example of downstream target of Ptf1a in the developing spinal cord. However, in contrast to Ptf1a-null mice, neurogenesis of Ptf1a-neurons is not affected in Neurog2 knock-out mice, raising the possibility that Ptf1a downstream targets other than Neurog2 are also involved in the development of Ptf1a-neurons. Moreover, Nephrin and Neph3 were more specifically expressed in the Ptf1a-positive domains, implicating their specific function in Ptf1a-neurons (7, 40). Compared with Neurog2, Nephrin and Neph3 as transmembrane proteins more likely mediate the effect of Ptf1a by directly influencing the cellular behavior of Ptf1a-neurons. Moreover, Nephrin and Neph3 were more specifically expressed in the Ptf1a-positive domains, implicating their specific function in Ptf1a-neurons (7, 40).

Ptf1a required co-factors for binding to the promoter region of Nephrin and Neph3 and to induce full promoter activation (Fig. 3 and supplemental Fig. 1). Ptf1a might also control Nephrin/Neph3 expression outside the nervous system, such as islet cells in the pancreas (20, 41–43).

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The function of Nephrin and Neph3 in the development of Ptf1α-neurons has yet to be determined. So far, no anatomical or morphological abnormalities have been demonstrated in the brains of NephrinA-null mice, and knock-out mice of NephrinB and Neph3 have not been reported (20). Neph3 is transiently expressed in early postmitotic neurons of the developing spinal cord, and intracellularly Neph3 is localized at the adherens junction between apical processes (17). Miyata et al. (47) reported that early postmitotic neurons maintain their apical processes before departing the ventricle to undergo radial migration. Therefore, one may speculate that Neph3 mediates the adhesion between the Ptf1α-progenitors, which in turn is necessary for their later development, such as neuronal migration, maturation, or differentiation. Alternatively, Neph3 trans-homophilic interaction may induce intracellular signals required for further development of Ptf1α-neurons. The ability of Neph family members in triggering intracellular signaling pathway has been best demonstrated in the case of Neph1. Neph1 overexpression activates the AP-1 promoter, and this activation is further stimulated by phosphorylation of Neph1 by tyrosine kinases (12, 48). Moreover, clustering of Neph1 results in its own tyrosine phosphorylation and its interaction with cytoplasmic proteins (49). Interestingly, the same study showed that Neph1 clustering elicits tyrosine phosphorylation of NephrinA and the recruitment of cytoplasmic proteins to the NephrinA complex (49). If Neph3 acts similarly to Neph1, we may speculate that in Ptf1α-neurons, Neph3 clustering triggered by its trans-homophilic binding may induce intracellular signals both via its own cytoplasmic domain and via its cis-interaction with NephrinA in the same cells.
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ular mechanism underlying Ptf1a-controlled neuronal cell fate specification must await clarification of signaling cascades downstream of Nephrin and Nephr3 and functional characterization of two isoforms of Nephrin.

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