The paired-like homeodomain protein, Arix, mediates protein kinase A-stimulated dopamine \( \beta \)-hydroxylase gene transcription through its phosphorylation status*

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The homeodomain transcription factor Arix/Phox2a plays a critical role in the specification of noradrenergic neurons by inducing the expression of dopamine \( \beta \)-hydroxylase (DBH), the terminal enzyme for noradrenaline biosynthesis. In reporter assays, Arix together with activation of cAMP-dependent protein kinase (PKA) potentiates DBH gene transcription. We have evaluated whether post-translational modification of Arix regulates PKA-mediated DBH gene transcription. We found that Arix is constitutively phosphorylated in vivo at the basal level and that the phosphorylation level is substantially decreased upon stimulation of the PKA pathway. The change in the Arix phosphorylation state coincides with DNA binding activity of Arix. Treatment of cells with forskolin results in a robust enhancement of the DNA binding of Arix, which is reversed by treatment with serine/threonine and tyrosine phosphatase inhibitors. Consistent with the DNA binding activity of Arix, treatment of cultured cells with phosphatase inhibitors diminishes transcriptional activation with Arix plus forskolin. Amino acid analysis demonstrates the presence of phosphoryl groups within Arix. The results collectively suggest that dephosphorylation of Arix is a necessary event to fully activate PKA-mediated DBH transcription. Thus, the present study demonstrates that Arix can integrate extrinsic signals through post-translational modification, regulating DBH gene transcription in response to activation of the PKA pathway.

In the central and peripheral nervous systems, identity of neurotransmitter is a pivotal attribute for proper function of a neuron. Neurotransmitter phenotype is a result of the activity of a neuron to synthesize, release, and uptake the neurotransmitter. Therefore, the expression of a biosynthetic enzyme and/or vesicular transporter in its given neuron defines a neurotransmitter identity. A coordinated expression of genes ultimately defines the phenotype of any neuron. Indeed, many transcription factors have been shown to play a key role in deciding the fate of a progenitor cell and in linking the cell to a variety of extracellular stimuli during development. Transcription factors of the homeodomain and basic-helix-loop-helix classes of proteins are frequently involved in the determination of cell type specificity (1, 2). Noradrenergic neurons are characterized by the coexpression of the catecholamine biosynthetic enzymes, tyrosine hydroxylase (TH)\(^1\) and DBH. TH, the rate-limiting enzyme of catecholamine biosynthesis, catalyzes the production of dihydroxyphenylalanine, which is in turn converted to dopamine by amino acid decarboxylase. DBH is the terminal enzyme that produces noradrenaline from dopamine; thus, the expression of DBH is essential for determination of noradrenergic cells. For specification of noradrenergic neurons, the paired-like homeodomain transcription factors, Arix/Phox2a and NBPhox/Phox2b, appear to act in concert to regulate noradrenergic traits in both the central and peripheral nervous systems (3). Arix and NBPhox are closely related homeodomain proteins that share significant amino acid sequence homology, including 100% identity in the homeodomain and 50% identity in the N terminus to the homeodomain. Coordinate action of Arix and NBPhox is essential for the proper development of central and peripheral noradrenergic cells. Targeted deletion analyses of Phox2a (4–6) and Phox2b (7) demonstrate the necessity of these genes to direct noradrenergic neuronal differentiation. Despite this implication, forced expression of Phox2a is only able to induce the expression of TH but not DBH in mammalian neural crest stem cell cultures. Importantly, the expression of both TH and DBH is evoked by Phox2a together with bone morphogenetic protein 2 (BMP2) and forskolin, which increases intracellular cAMP levels (8). In contrast, in chicken and zebrafish, forced expression of Phox2a is sufficient to promote the generation of ectopic noradrenergic neurons that express TH, DBH, and pan-neuronal genes (4, 9, 10). These experimental findings in vivo and in ovo suggest that Phox2a requires an additional factor and/or an environmental stimulus that is present in the embryo in order to potentiate activation of target genes.

Phox2a expression is maintained in adult noradrenergic cells, where it likely functions to sustain the expression of the genes necessary for noradrenaline biosynthesis. The transcription of TH and DBH is regulated postnatally by environmental stimuli, such as stress, which trigger intracelluar signaling pathways (11). The interaction between Phox2a and signaling molecules is likely to extend beyond neuronal development and function to modulate neurotransmitter biosynthesis in the

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1 The abbreviations used are: TH, tyrosine hydroxylase; DBH, dopamine \( \beta \)-hydroxylase; PKA, cAMP-dependent protein kinase; rPKA, recombinant PKA; CREB, cAMP-response element-binding protein; CBP, CREB-binding protein; HA, hemagglutinin; EMSA, electrophoretic mobility shift assays; MAP, mitogen-activated protein kinase; IVT, in vitro translated; BMP, bone morphogenetic protein; PP2A, phosphatase 2A; SCR, Sex Combs Reduced.
adult as well. In addition to its function in neurotransmitter identity, Arix/Phox2a may also play a critical role in the development of midbrain motor nuclei. A mutation in the N-terminal region of human ARIX results in congenital fibrosis of the extraocular muscles type 2, believed to result from the maldevelopment of cranial nerve nuclei nII, nIV, and nVI (12).

In addition to the genetic manipulation studies, we and others (13–15) have demonstrated a direct link between Phox2 transcription factors and DBH gene expression by analyses of the 5′-upstream promoter of the DBH gene. In previous studies, we found three homeodomain protein recognition sites (HD1, -2, and -3) for Arix and NBPhox within the proximal rat DBH promoter. Reporter assays using mutant promoters showed that all three HD sites are integrated and interdependent in the regulation of DBH transcription. Two of the homeodomain protein recognition sites of the rat DBH promoter, HD1 and HD2, lie within a genetic regulatory element, DB1, that mediates PKA responsiveness, noradrenergic tissue specificity, and Arix-dependent activation of DBH transcription (13, 16). Adjacent to sites HD1 and -2 on the DB1 enhancer is a CAMP-response element/activator protein 1 (CRE/AP1) site. Enhancement of DBH transcription by second messengers occurs in catecholaminergic cell lines expressing endogenous DBH and is mediated through the CRE/AP1 element on the DBH promoter (17). Binding of AP1 family proteins, including c-Fos and c-Jun, to the CRE/AP1 site is necessary to achieve transcriptional synergism of Arix with the PKA pathway (17). In addition, recruitment of the coactivator, CRE-binding protein (CBP), appears to be involved in the DBH promoter activation by Arix and PKA (18). CBP physically interacts with the transcription domain of Arix and augments Arix-mediated DBH transcription in a PKA-dependent manner. However, it is unknown how Arix acts through multiple HD sites to integrate the activation of the PKA pathway. One plausible mechanism would involve post-translational modification of Arix in response to PKA activation.

Protein phosphorylation is an important post-translational modification to modulate the function of proteins in response to the extracellular stimuli. In general, the phosphorylation state of a transcription factor may influence its activity by modifying nuclear translocation, DNA binding, or transactivation potential (19, 20). A few cases of homeodomain proteins whose function is regulated by phosphorylation have been reported. The Cut homeodomain transcription factor is phosphorylated in vitro by casein kinase II and protein kinase C in the Cut repeats, a DNA-binding motif in addition to the homeodomain, causing a reduction in DNA binding and transcriptional repression (21, 22). Casein kinase II is also known to phosphorylate the homeodomain of Csx/Nkx2.5, increasing DNA binding (23). PKA can directly phosphorylate the homeodomain of Oct-1 in vitro in a mitosis-specific manner (24). Evidence of phosphorylation of homeodomain proteins further extends to functional relevance in vivo. Regulated phosphorylation of Drosophila Antennapedia by casein kinase II plays an important role in the appropriate development of thorax and abdomen during embryogenesis (25). These developmental influences appear to result from the ability of Antennapedia to bind DNA cooperatively with another homeodomain protein, Extradenticle, upon phosphorylation of Antennapedia.

Natural target genes for most of the homeodomain proteins are unknown; therefore, little is known about the transcriptional mechanisms that achieve their functional specificity. In the present study, we took advantage of the known in vitro target gene for Arix, and we have characterized the molecular mechanism of Arix in the activation of DBH gene transcription in coordination with activation of the PKA pathway. We found that Arix is a constitutive phosphoprotein and that the phosphorylation state of Arix is dramatically decreased in response to activation of the PKA pathway. PKA activation leads to increased DNA binding activity of Arix, which is abolished by pretreatment with phosphatase inhibitors. Collectively, the present study suggests that a dephosphorylated form of Arix is transcriptionally active and that the regulated post-translational modification is a crucial event to functionally activate Arix in response to an extracellular stimuli. This is the first demonstration of functional regulation of a paired-type homeodomain protein by phosphorylation.

MATERIALS AND METHODS

Cell Culture—HepG2 cells were cultured in minimum Eagle’s medium supplemented with 10% fetal bovine serum (HyClone), 1% non-essential amino acids, and 110 mg/liter sodium pyruvate. HEK293 cells were cultured in Dulbecco’s modified Eagle’s medium with an addition of 10% fetal bovine serum. CATH.a cells were cultured in RPMI plus 8% horse and 4% fetal bovine sera. All cell lines were maintained at 37 °C in an atmosphere of humidified air containing 5% CO2.

Plasmid Constructs—The construction of DBH-Luc (−232/+10) reporter plasmid containing the promoter and 5′-flanking sequence of DBH promoter was described previously (18). Briefly, the reporter construct contains the proximal DBH promoter (−232/+10) including the DB1 enhancer region, TATA-like sequence, and the transcription start site, linked upstream of the coding sequence of firefly (Photinus pyralis) luciferase. The RSV-PKA expression construct, containing cDNA for the catalytic subunit of PKA, was a generous gift from Dr. Richard Maurer (Oregon Health Sciences University, previously described by Maurer (26)). Hemagglutinin (HA)-tagged full-length (HA-Arix) and truncated Arix (Ar-N, Ar-C, and HDAr) expression plasmids were constructed as described by Adachi et al. (14).

Transfections and Reporter Assays—DNA used for transfections was purified by equilibrium centrifugation in CsCl-ethidium bromide gradients. For HepG2 cell transfections, cells were plated at a density of 0.25 × 106 cells per well in a 6-well plate 1 day before transfection and transiently transfected with DBH-Luc (750 ng), HA-Arix (100 ng), and pRL-null (100 ng) by calcium phosphate precipitation as described previously (16). Similarly, CATH.a cells were transfected using Genefect (Venn Nova, Inc.), according to the manufacturer’s recommendations. The total amounts of DNA were adjusted to 2 μg using HAB.1, the backbone of an expression vector. Cells were harvested 24 h after transfection, and aliquots of cell extracts were assayed for protein content and lucerase activity using Dual-Lucerase Assay System (Promega). For some experiments, cells were stimulated with forskolin at a concentration of 20 μM for 7 h before harvest. Orthovanadate (1 mM final concentration) or okadaic acid (1 μM or 50 mM final concentration) treatments were carried out for 30 min prior to the forskolin stimulation. As an internal control of transfection efficiency, all transfections contained a jellyfish luciferase (Renilla) plasmid that lacks a promoter (pRL-null). Values presented for reporter gene activity are standardized to Renilla luciferase activity per extract.

In Vitro Kinase Assays—For substrates, in vitro translated and 32P-labeled Arix, truncated Arix constructs, and NBPhox were produced using TNT-coupled wheat germ extract system (Promega). Phosphorylation of substrates were carried out at 30 °C for 30 min in an ATP-regenerating kinase buffer system. The kinase buffer includes 0.5 μl of 10 mM Tris-Cl (pH 8), 2 mM MgCl2, 5 mM ATP, 10 mM phosphocreatine, 3.5 units/ml creatine kinase, 2.5 μM okadaic acid, 0.5 mM sodium orthovanadate, and either 10 μg of casein, cell nuclear extracts or 10 ng of purified and recombinant catalytic subunit of PKA (rPKA) as a source of kinases. In some kinase reactions, 5 μg of a PKA-specific inhibitor, H-89, was included during incubation. In dephosphorylation experiments, 0.06 unit of potato acid phosphatase (Roche Molecular Biochemicals) was added in the kinase reaction mixture. The reaction products were analyzed by SDS-PAGE, followed by autoradiography. For the in vitro kinase assay using γ[32P]ATP, bacterially produced and purified His-tagged Arix proteins (1 μg) (14) were incubated at 30 °C for 5 min with 10 ng of rPKA in the kinase reaction buffer containing 12.5 mM Tris-Cl (pH 8), 0.1 mM ATP, 10 mM MgCl2, 0.25 mg/ml bovine serum albumin, and 0.5 μCi γ[32P]ATP. The reaction was stopped by adding EDTA to the final concentration of 80 mM on ice. As control reactions, wild-type and mutant CREB proteins (0.5 μg each) were used as substrates. The reaction products were separated on a SDS-PAGE gel and autoradiographed. rPKA and wild-type and
In Vivo Phosphorylation Studies—3.5–4 × 10⁶ of HEK293 cells were seeded on a 100-mm polylysine-coated plate 1 day before transfection. 10 μg of DNA was transfected using LipofectAMINE (Invitrogen) according to the manufacturer’s instructions. The total amount of DNA was adjusted with HA1. The following day, cells were incubated in phosphate-free Dulbecco’s modified Eagle’s medium containing 500 μCi/mL of [32P]orthophosphate for 4 h. Cells were harvested and lysed in RIPA buffer (50 mM Tris-Cl, pH 8), 150 mM NaCl, 1 mM EDTA, 0.5% deoxycholate, 1% Nonidet P-40, 0.1% SDS), and lysates were cleared by centrifugation. HA-tagged Arix was immunoprecipitated with HA antibody (3F10, Roche Molecular Biochemicals) and resolved on 10% SDS-polyacrylamide gel, followed by autoradiography. For quantitation of 32P incorporation, a gel was exposed to a PhosphorImager (Molecular Dynamics), and 32P signals were quantitatively analyzed.

Two-dimensional Phosphoamino Acid Mapping Analyses—32P-Labeled proteins were excised from SDS-PAGE gels and ground in 1 ml of 50 mM ammonium bicarbonate (pH 7.3) solution including 10 μl of 10% SDS and 50 μl of β-mercaptoethanol. The samples were boiled for 5 min and eluted with shaking at room temperature for 3 h twice. Eluted proteins were precipitated by 250 μl of trichloroacetic acid on ice for 1 h with 40 μg of RNase A as a carrier protein and collected by centrifugation. Resultant pellets were washed with ice-cold 100% ethanol, air-dried and subjected to hydrolysis in 5.7 mM HCl for 1 h at 110°C. After hydrolysis, samples were lyophilized in a Speed-Vac and resuspended in 5–10 μl of pH 1.9 buffer (88% formic acid, acetic acid, and deionized water at a ratio of 50:156:1794 [v/v]), which contains phosphoamino acid standards. Samples were then electrophoresed on a cellulose thin layer plate in the pH 1.9 buffer at 1.5 kV for 20 min as the first dimension, followed by the second dimension electrophoresis in pH 3.5 buffer consisting of acetic acid, pyridine, and deionized water (100:10:1890 [v/v/v]) at 1.3 kV for 16 min. The plate was completely dried, sprayed with 0.25% ninhydrin solution in acetone to visualize the standards, and autoradiographed.

In Vitro Protein-Protein Interaction Assay—His pull-down assays and micrococcal nuclease treatment were carried out as described previously (14).

HEK293 Cell Extracts and Electrophoretic Mobility Shift Assays (EMSA)—To prepare cell extracts used for EMSA, HEK293 cells were plated at a density of 3.5 × 10⁶ cells in a 100-mm polylysine-coated plate (EMSA)–the next day using LipofectAMINE (Invitrogen) according to the manufacturer’s instructions. During transfection, HEK293 cells were incubated in Opti-MEM (Invitrogen) with a lipid-DNA mixture at a ratio of 3:1 for 5 h. The following day, cells were treated with forskolin (20 μM at a final concentration) or vehicle for 1 h before harvest. Some of them were pretreated with okadaic acid (50 nM) or orthovanadate (1 mM) for 30 min prior to the forskolin stimulation. Harvested cell pellets were resuspended in 50–100 μl of the RIPA buffer and incubated for 20 min on ice. The cell extracts were collected by centrifugation, and the protein concentration was assayed by Bradford (Bio-Rad). To quantitate the amount of Arix in cell extracts, 2.5 μg of cell extracts were separated on 10% SDS-polyacrylamide gel and subjected to Western blot analyses using HA antibody (3F10, Roche Molecular Biochemicals). EMSA was carried out using 0.1 μg of HEK293 cell extracts as described previously (14). For the quantitation of EMSA, relative proportions of bound probes were calculated using a PhosphorImager (Molecular Dynamics).

RESULTS

Arix Is Post-translationally Modified in Vitro and in Vivo—We have demonstrated previously (13) that Arix can produce a modest activation of transcription from the rat DBH promoter, and that the transcriptional activity of Arix is greatly potentiated by stimulation of the PKA pathway. In previous studies (17), we found that PKA-dependent DBH transcription is associate with recruitment of Fos and Jun to the CRE/AP1 element on the DBH promoter. If binding of Fos and Jun to the DBH CRE/AP1 element is sufficient for activation of transcription by PKA, we would anticipate that introduction of Fos and Jun into cells would mimic the effect of PKA on DBH transcription. To analyze this hypothesis, Fos and Jun expression constructs were cotransfected with the DBH promoter-reporter construct, DBH-luc, plus an Arix expression construct.
To evaluate whether PKA was the kinase phosphorylating Arix in vitro, the PKA-specific inhibitor, H-89, was added to the reactions. However, H-89 did not eliminate mobility shifts created by PC12 nuclear extracts in both Arix and NBPhox, nor did a direct application of rPKA cause the appearance of mobility shifts. We also tested for direct incorporation of \[^{32}P\]ATP into bacterially produced recombinant Arix by rPKA. rPKA efficiently phosphorylated recombinant CREB protein as demonstrated by \[^{32}P\] incorporation but not a mutant form of CREB, which lacks PKA phosphorylation sites at serine 133 residue (Fig. 2B). Recombinant Arix exhibited no significant \[^{32}P\] incorporation by rPKA. These results strongly suggest that the phosphorylation of Arix and NBPhox is not a direct action of PKA.

Next, we examined whether Arix is phosphorylated in vivo by metabolically labeling cells with \[^{32}P\]orthophosphate. The Arix cDNA construct was tagged with hemagglutinin antigen (HA), and HEK293 cells were transiently transfected with HA-Arix, followed by immunoprecipitation with an HA antibody. HEK293 cells were chosen for these experiments because they have a much higher transfection efficiency (~80%) than HepG2 cells, which have only 2–3% transfection efficiency. Furthermore, similar to HepG2 cells, HEK293 cells lack endogenous expression of Arix and demonstrate the activation of the DBH promoter by Arix and PKA; together Arix and the activation of PKA potentiated DBH transcription compared with Arix or PKA alone (Fig. 2C). When HEK293 cells were transiently transfected with HA-Arix expression vector and metabolically labeled with \[^{32}P\]orthophosphate, HA-Arix was immunoprecipitated with an HA antibody and resolved on 10% SDS-PAGE, followed by autoradiography (middle panel as indicated by \[^{32}P\]). Immunoprecipitated Arix was also subjected to Western blot and stained with HA antibody (bottom panel as indicated by aHA) to examine the amount of Arix. \[^{32}P\] incorporation was quantitatively analyzed using a PhosphorImager. Changes in \[^{32}P\] incorporation are expressed as a percentage of Arix phosphorylation under the basal condition (top panel). Bars represent mean values ± range from two independent experiments.

Fig. 2. Arix is phosphorylated both in vitro and in vivo. A, \[^{35}S\]-labeled IVT Arix (left panel) or NBPhox (right panel) was subjected to the in vitro kinase assay using PC12 nuclear extracts or rPKA as a source of kinases. Some assays included 5 μM of H-89, a PKA-specific inhibitor. Potato acid phosphatase (PAPase) was added to dephosphorylate the phosphorylated products; therefore, PAPase-sensitive mobility shifts are evidence of phosphorylation. B, bacterially produced Arix, wild-type CREB (WT), and mutant CREB (mt) were incubated in the kinase reaction buffer with rPKA in the presence of \[^{32}P\]ATP. The amounts of Arix and CREB loaded in each lane are approximately equal as examined by Coomassie Blue staining of a gel (data not shown). C, DBH promoter activity was examined in HEK293 cells either with or without the expression vectors containing Arix or the PKA catalytic subunit (250 ng). D, HEK293 cells were transiently transfected with 5 μg of HA-Arix plasmid either with or without 2.5 μg of the PKA expression plasmid and metabolically labeled with \[^{32}P\]orthophosphate. HA-Arix was immunoprecipitated with an HA antibody and resolved on 10% SDS-PAGE, followed by autoradiography (middle panel as indicated by \[^{32}P\]). Immunoprecipitated Arix was also subjected to Western blot and stained with HA antibody (bottom panel as indicated by aHA) to examine the amount of Arix. \[^{32}P\] incorporation was quantitatively analyzed using a PhosphorImager. Changes in \[^{32}P\] incorporation are expressed as a percentage of Arix phosphorylation under the basal condition (top panel). Bars represent mean values ± range from two independent experiments.

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Protein-Protein Interactions of Arix and NBPhox Are Favored in Dephosphorylated States—Previously, we reported that Arix

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and NBPhox have the ability to form homo- and heterodimers in vitro (14) and in vivo (18). Protein-protein interactions between Arix and NBPhox at the multiple homeodomain recognition sites on the DBH promoter may be a mechanism to regulate DBH transcription. To seek the functional relevance of the phosphorylation event of Arix, we examined whether there is preference in either the phosphorylation or dephosphorylation state of Arix and NBPhox for protein-protein interactions. First, 35S-labeled IVT proteins were subject to the kinase reaction with PC12 nuclear extracts to generate phosphorylated proteins as described above (Fig. 3A). 35S-Labeled IVT proteins containing phosphorylated products were then used for His pull-down assays. In these assays, protein-protein interactions were monitored by measuring the ability of 35S-labeled IVT proteins to coprecipitate with His-Arix or His-NBPhox proteins conjugated to nickel resin. His-Arix or His-NBPhox interacting proteins were resolved on SDS-PAGE and detected by 35S signals. As shown in Fig. 3B, 35S-labeled IVT products did not directly bind to the metal resin. His-Arix interacting proteins were primarily the faster migrating proteins from both IVT-Arix and NBPhox, presumably dephosphorylated proteins (Fig. 3B). In contrast, an analysis of an aliquot of supernatant after the binding reaction demonstrated the presence of slower migrating proteins, suggesting that unbound fractions are mostly phosphorylated products. Consistently, in a reciprocal experiment using His-NBPhox conjugated to nickel resin, His-NBPhox interacting proteins were mostly in a dephosphorylated form of Arix and NBPhox. To eliminate the possibility that these protein-protein interactions are non-specifically mediated by association with DNA, the precipitates were further treated with micrococcal nuclease to digest nonspecific DNA prior to SDS-PAGE analyses (Fig. 3C). Even after the micrococcal treatment, the protein-protein interactions were evident. These protein-protein interaction studies imply that dephosphorylated rather than phosphorylated Arix and NBPhox favorably undergo physical interactions.

PKA-dependent Dephosphorylation of Arix May Be a Key Step to Activate DBH Transcription—Within the DBH promoter,
Role of Arix Phosphorylation in DBH Transcription

there are three homeodomain recognition sites (HD1–3) (Fig. 4A). Recombinant Arix and NBphox can bind to the DBH promoter through the HD1–3 sites (14), and all three sites are functionally required to regulate DBH transcription (18). Based on our previous findings, we used oligonucleotides containing these sites, named DB1 and HD3 probes (Fig. 4A), to examine whether the DNA binding activity of Arix is altered upon activation of the PKA pathway. EMSAs were carried out using cell extracts from HEK293 cells transiently transfected with the HA-Arix expression vector (Fig. 4B). Some cells were stimulated with forskolin to activate the PKA pathway and pretreated with phosphatase inhibitors, okadaic acid, or orthovanadate, prior to the preparation of the extracts. By using the HD3 probe, Arix-containing extract exhibited a supershift with an HA antibody. The formation of the supershift was robustly increased in the EMSA reaction with the Arix-containing extract prepared from forskolin-treated cells. The supershift formation was absent in the mutant probe, HD3m, where the HD site is mutated, demonstrating that the supershift formation is dependent on the HD sites. Similar to our observation, others (27) have also observed that the signal from the Arix-DNA-antibody complex is stronger than that from the Arix-DNA complex. Thus, supershift formation is often used as a way to identify Arix-containing specific DNA-protein complexes and the extent of supershift formation reflects the DNA binding activity of Arix. Stimulation of the PKA pathway consistently results in a substantial increase in the DNA binding activity of Arix, from 2.5- to 3.5-fold between experiments, as quantitated by the intensity of the supershift.

Because Arix undergoes dephosphorylation upon PKA stimulation, as shown by in vivo ³²P labeling experiments (Fig. 2C), we asked whether phosphatase inhibitors can reverse the effect of PKA activation on the DNA binding activity of Arix. Arix-transfected cells were pretreated with okadaic acid or orthovanadate as serine/threonine and tyrosine phosphatase inhibitors, respectively, followed by stimulation of PKA activity by forskolin. In these phosphatase inhibitor-treated cells, the formation of supershift significantly decreased, demonstrating weakened DNA binding activity of Arix (Fig. 4B). The extent of the supershift formation was reversed close to the basal state of Arix.

We also examined the effect of PKA stimulation on the DNA binding activity of Arix using the DB1 probe, which contains two HD sites. Similar to the results from EMSA reactions using HD3 probe, the Arix-containing extract from forskolin-treated cells demonstrated a robust supershift formation, which was almost absent in forskolin-untreated cells (Fig. 4B). The supershift was no longer present in the mutant probe, 2HDm, confirming the specificity of the supershift formation through the HD sites. When the intensity of supershift was quantitated, extracts from forskolin-treated cells exhibited a 2–6-fold increase in supershift formation between experiments. The phosphatase inhibitor treatment using okadaic acid as well as orthovanadate exhibited a reduction in the supershift formation, consistent to the EMSA reactions using the HD3 probe.

The specificity of the supershift appearing upon the addition of HA antibody was further characterized using a probe (−180/−14) containing all three Arix-binding sites (HD1–3). The supershift was specifically competed by either the DB1 or HD3 oligonucleotide, but not by oligonucleotides containing mutations in the HD sites (Fig. 4C). These results confirm that the supershift formation is specific to Arix-binding sites of the DBH promoter. Changes in the intensity of supershift formation were not because of different contents of Arix in cell extracts as indicated by the Western blot (Fig. 4D), suggesting that post-translational modification of Arix by the PKA pathway alters the DNA binding activity.

The results from EMSA analyses imply that the DNA binding activity of Arix is enhanced by a dephosphorylation event resulting from stimulation of the PKA pathway. To evaluate this model in cell culture, the effect of phosphatase inhibitors on transcription from the DBH promoter-reporter construct, DBH-Luc, was evaluated in CATH.a cells, a catecholaminergic cell line, which expresses endogenous DBH and Arix genes.
Role of Arix Phosphorylation in DBH Transcription

To understand further the mechanism involved in the PKA-regulated activity of Arix, we have begun to define the phosphorylation sites on Arix responsible for the functional changes in DNA binding. Analyses of the primary amino acid sequence of Arix for consensus phosphorylation sites revealed that multiple phosphorylation sites are present in the C terminus as well as in the homeodomain but none in the N terminus (Fig. 6A). These sites are potential targets for tyrosine kinase, casein kinase II, protein kinase C, and mitogen-activated protein (MAP) kinase. No PKA phosphorylation sites were predicted, consistent with the results presented in Fig. 2, A and B. To map the phosphorylation sites of Arix, we first performed in vitro kinase assays using truncated Arix constructs (Fig. 6A) as substrates and PC12 cell nuclear extracts as a source of kinases. Phosphatase-sensitive mobility shifts, indicative of phosphorylated form of proteins, were observed from Ar-N and HDAr constructs, consistent with the prediction that phosphorylation sites reside in the homeodomain (Fig. 6B). However, the Ar-C construct did not exhibit phosphatase-sensitive mobility shifts, even though the in vitro kinase assay with the homeodomain alone resulted in mobility shifts. It is possible that the C-terminal truncation caused a structural change that masked the phosphorylation sites; therefore, phosphatase-sensitive shifts were undetectable in this in vitro kinase assay system.

Previous experiments (14) have demonstrated that truncation of the C terminus does not reduce the transcriptional synergism between PKA and Arix on the DBH promoter activity. Therefore, we examined the phosphorylation state of the Ar-C construct by in vivo 32P labeling. In vivo phosphorylation of the Ar-C construct was apparent (Fig. 6C) although 32P incorporation was reduced by 40–90% between two experiments, compared with full-length Arix. The reduction in 32P incorporation of the Ar-C construct can be attributed to the fact that multiple phosphorylation sites are present in the C-terminal segment, whereas fewer phosphorylation sites are located in the homeodomain and/or the N terminus of Arix.

To ascertain which amino acids are phosphorylated, 32P-labeled Arix proteins were extracted from the gel shown in Fig. 6C and subjected to a phosphoamino acid mapping. In this analysis, phosphoserine was evident from both the full-length and C-terminal truncated Arix proteins (Fig 6D). This result suggests that not only are there sites for serine kinases in the C terminus, as predicted by consensus sequence analyses, but there are additional phosphorylation sites on serine in the homeodomain and/or the N terminus, not predicted by the sequence analyses. However, we were not able to observe detectable signals for either phosphotyrosine or phosphothreonine using either full-length Arix or Ar-C proteins. Taken together, we conclude that there are multiple serine phosphorylation sites in Arix, one or more of which plays a regulatory role in Arix function.
The regulation of transcription from the rat noradrenergic neurons in developing nervous systems (8, 18). The present study gives insight into the molecular mechanisms underlying the transcriptional activity of Arix in coordination with the cAMP/PKA signaling pathway in the activation of DBH transcription. We demonstrate that Arix is constitutively phosphorylated at multiple serine residues in cell culture. Stimulation of the PKA pathway leads to a significant decrease in the phosphorylation state of Arix, which coincides with increased DNA binding activity of Arix to the multiple HD sites within the DBH proximal promoter. The increase in DNA binding activity of Arix is abolished by phosphatase inhibitor treatment. Consistently, the phosphatase treatment reduces the transcriptional activity of Arix in coordination with the cAMP/PKA signaling pathway in the activation of DBH transcription. We demonstrate that Arix is constitutively phosphorylated at multiple serine residues in cell culture. Stimulation of the PKA pathway leads to a significant decrease in the phosphorylation state of Arix, which coincides with increased DNA binding activity of Arix to the multiple HD sites within the DBH proximal promoter. The increase in DNA binding activity of Arix is abolished by phosphatase inhibitor treatments. Consistently, the phosphatase treatment reduces the PKA-stimulated DBH promoter activity mediated by Arix. Taken together, the present findings imply that the transcriptional activity of Arix is negatively regulated by phosphorylation and that activation of the PKA pathway causes dephosphorylation of Arix, converting it to a transcriptionally competent form, and potentiating DBH gene transcription.

**Possible Kinases and Phosphatases That Regulate Phosphorylation of Arix**—The regulation of transcription from the rat DBH promoter by Arix plus activation of the PKA pathway entails multiple events. Recruitment of AP1 proteins, Fos and Jun, to the CRE/AP1 site is a part of the mechanism of PKA-stimulated DBH transcription (17), and the transcriptional coactivator, CBP, a signal-dependent facilitator of PKA-stimulated DBH transcription (18) is also involved. The present results further extend the molecular mechanisms of DBH gene transcription by demonstrating that the post-translational modification of Arix is regulated by PKA and influences its DNA binding activity. Consistent with the fact that phosphorylation of Arix is constitutive under basal conditions, PKA does not directly phosphorylate Arix.

The kinase responsible for phosphorylation of Arix, as well as the regulated phosphorylation site, remains to be determined. The identification of the kinase and regulated phosphorylation site is likely to be complicated, because the experiments herein suggest multiple phosphoserine sites, and yet, using several search programs, no consensus sites for phosphoserine were identified within the appropriate region of the protein. The regulated phosphorylation site should occur within the N-terminal or homeodomain regions of Arix, because these domains are sufficient to interact synergistically with PKA in the activation of DBH transcription (14). Nonetheless, there are 9 serine residues in the N-terminal segment of Arix and 1 in the homeodomain (Fig 7A). Candidate kinases may be one of the proline-directed kinases, such as glycogen synthase kinase or MAP kinases, because the N-terminal section of Arix, which contains the transcriptional activation domain, is proline-rich, with serines embedded within the proline-rich segments. Further suggestion of the involvement of MAP kinases as negative regulators of DBH transcription arises from studies using leukemia inhibitory factor and ciliary neurotrophic factor. These factors will induce a neurotransmitter-phenotype switch in sympathetic neurons from noradrenergic to cholinergic (28). During the phenotype switch, leukemia inhibitory factor and ciliary neurotrophic factor cause down-regulation of DBH gene transcription, which can be blocked by a MAP kinase inhibitor, PD98059 (29). This observation suggests that activation of MAP kinase negatively regulates DBH transcription. It is plausible that the growth factors present in the local environment of a cell maintain a basal level of MAP kinase, which, in turn, phosphorylates Arix. When cells encounter another environmental cue that activates the PKA pathway, phosphorylated Arix is converted to a dephosphorylated form, gaining stronger transcriptional activity and enhancing DBH transcription.

The phosphatase that dephosphorylates Arix in response to PKA stimulation is also not identified, although protein phosphatase 2A (PP2A), a serine/threonine phosphatase, is an attractive candidate. A primary target of okadaic acid is PP2A (30), and thus the ability of low concentrations of okadaic acid...
to inhibit the DNA binding activity of Arix and reduce PKA responsiveness suggests involvement of PP2A. The regulatory B subunit of PP2A determines the substrate specificity of the enzyme. In particular, B56/H9254 is highly expressed in brain and concentrated in the nucleus as a phosphoprotein in vivo (31).

Furthermore, in vitro studies suggest that phosphorylation of PP2A by PKA positively regulates its phosphatase activity (32). Increasing lines of evidence suggest that protein phosphatases play an important role in developmental processes (33, 34).

Partial inhibition of binding and transcriptional activation was also observed with the phosphotyrosine phosphatase inhibitor orthovanadate. Repeated phosphoamino acid analyses of phosphorylated Arix did not reveal phosphotyrosine, suggesting that the effect of vanadate may be indirect. A conserved tyrosine in the homeodomain (Tyr-25) was altered to phenylalanine by mutagenesis but did not produce a constitutively active transcription factor, as would be expected if it were the regulated phosphoamino acid (data not shown). The effect of vanadate suggests that PKA stimulates multiple phosphorylation/dephosphorylation cascades which affect Arix activity both by direct and indirect pathways.

**Dephosphorylation of Arix Positively Regulates Its Transcriptional Potential**—As demonstrated by EMSA (Fig 3), the DNA binding activity of Arix is greatly enhanced by stimulation of the PKA pathway, which appears to lead to dephosphorylation of Arix. In addition, serine/threonine as well as tyrosine phosphatase inhibitor treatments abrogated the enhanced DNA binding of Arix, suggesting that phosphorylation at both serine/threonine and tyrosine residues play an inhibitory role in DNA binding. **In vitro** studies suggest that the homeodomain can be phosphorylated. In principle, phosphorylation in the DNA binding domain introduces a negative charge, which is unfavorable for DNA binding because the backbone of DNA is negatively charged in character. If phosphorylation occurs within or close to the homeodomain of Arix, removal of phosphorylation would be an obligatory step to confer DNA binding activity of Arix. With regard to the homeodomain proteins, Berry and Gehring (35) reported that the Drosophila Hox protein, Sex Combs Reduced (SCR), which determines the identity of specific segments, is regulated by phosphorylation. **In vivo** analyses using transgenic flies revealed that a mutant form of SCR mimicking constitutive phosphorylation in the N-terminal arm of the homeodomain is functionally inactive. Dephosphorylation is apparently necessary to switch SCR in an active state, possibly by PP2A, because a yeast two-hybrid screen revealed the interaction between SCR and PP2A.

The functional importance of the phosphorylation state of Arix is also suggested by **in vitro** protein-protein interaction assays (Fig. 3). Homo- and heteromerization of Arix and NBPhox occurs preferably in the dephosphorylated forms of these proteins, rather than the phosphorylated forms. Whether binding of Arix to DNA requires protein dimerization is unknown at present. If this is the case, phosphorylation at the dimerization interface may decrease dimerization, thereby inhibiting DNA binding. Alternatively, because the DBH proximal promoter carries multiple homeodomain recognition sites, multimerization of Arix and NBPhox may confer cooperativity in DNA binding. Upon stimulation of the PKA pathway, dephosphorylated Arix may undergo multimer formation, stabilizing the transcriptional machinery at the promoter, thereby enhancing DBH transcription.

**Arix May Integrate Extracellular Signals through the cAMP/PKA Pathway to Enhance DBH Gene Transcription during Noradrenergic Differentiation and Environmental Stimulation**—During embryogenesis, noradrenergic differentiation is believed to be triggered by BMPs, secreted proteins of the trans-
forming growth factor β superfamily. Exposure to BMPs leads to the sequential induction of Mash1 and Phox2a/Arix expression (8, 37). However, induction of TH and DBH expression in mammalian neuronal crest stem cell culture requires the activation of PKA, through the elevation of intracellular cAMP (8), and cAMP potentiates the influence of BMPs in inducing TH expression in avian neural crest cultures (38). In other developmental processes such as renal branching morphogenesis (39) and chondrogenesis (40), BMP2 has been demonstrated to increase PKA activity. Therefore, it is plausible that BMP2/4, which have been shown to induce the expression of TH and DBH in vivo (8, 41), can stimulate the cAMP/PKA pathway in noradrenergic precursor cells.

Based on our results, the model for the regulation of DBH gene transcription is outlined in Fig. 7B. Arix, the noradrenergic tissue-specific transcription factor, is present in a constitutively phosphorylated form under the basal condition, which is a transcriptionally active state. Phosphorylated Arix has weak DNA binding activity that contributes to the minimal activation of DBH transcription. When cells encounter an environmental cue, the cAMP/PKA pathway is activated, which will dephosphorylate Arix at the homeodomain or the N terminus. Dephosphorylated Arix is in an active state by acquiring strong DNA binding activity. As a result, dephosphorylated Arix becomes competent to fully activate DBH transcription. This mechanism may be active during development or in mature noradrenergic cells. The present study demonstrates that the phosphorylation state of Arix shifts its functional activity through DNA binding and that Arix may serve as a signal-decoding molecular switch. Arix is present in a constitutively phosphorylated form under the basal condition, which is a transcriptionally reduced state. Phosphorylated Arix has strong DNA binding activity that contributes to the minimal activation of DBH transcription. When cells encounter an environmental cue, the cAMP/PKA pathway is activated, which will dephosphorylate Arix at the homeodomain or the N terminus. Dephosphorylated Arix is in an active state by acquiring strong DNA binding activity. As a result, dephosphorylated Arix becomes competent to fully activate DBH transcription. This mechanism may be active during development or in mature noradrenergic cells. The present study demonstrates that the phosphorylation state of Arix shifts its functional activity through DNA binding and that Arix may serve as a signal-dependent tissue-specific transcription factor that consolidates an extracellular cue, promoting a noradrenergic fate and regulating noradrenaline biosynthesis.

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