The cAMP Pathway Regulates Both Transcription and Activity of the Paired Homeobox Transcription Factor Phox2a Required for Development of Neural Crest-derived and Central Nervous System-derived Catecholaminergic Neurons*

Sigeng Chen, Ming Ji', Maryline Paris', Ronald L. Hullinger, and Ourania M. Andrisani*  
From the Department of Basic Medical Sciences, Purdue University, West Lafayette, Indiana 47906

The neural crest (NC) is a pluripotent cell population derived from the lateral ridges of the neuroepithelium during closure of the neural tube (1). NC cells migrate along defined paths in the developing embryo, generating diverse cell types (2). The sympathoadrenal (SA) lineage originating from the trunk region of the neural tube (1) in response to instructive, microenvironmental signals develops to sympathetic neurons and chromaffin cells of the adrenal medulla (3). Cells committed to the SA lineage are characterized by expression of tyrosine hydroxylase (TH), the rate-limiting enzyme in catecholamine biosynthesis, and dopamine-β-hydroxylase (DBH), catalyzing the conversion of dopamine to norepinephrine (3).

Bone morphogenetic proteins (BMPs) produced by the developing aorta (4–9) and unidentified signals originating from the notochord and ventral neural tube (10–14) are required for SA cell development. However, in murine (15) and avian (16, 17) NC cultures, SA cell development requires not only BMP2 but also moderate activation of cAMP signaling. Importantly, our earlier studies (16) demonstrated moderate activation of cAMP signaling synergizes with BMP2 in promoting development of the SA lineage. However, the molecular mechanism by which cAMP signaling in synergy with BMP2 promotes SA cell development is unknown.

The cAMP pathway (18), via the cAMP-dependent protein kinase A (PKA), activates the transcription factor CREB by Ser-133 phosphorylation (19, 20). Phospho-CREB interacts with the coactivator CBP/p300, mediating cAMP-dependent transcription (21). CREB null mice (22) demonstrate CREB-dependent, neurotrophin-mediated survival and growth of peripheral neuronal neurons as well as a 65% CREB-dependent reduction in superior cervical ganglia sympathetic neuron development, occurring before neurotrophin dependence. These results implicate CREB in sympathetic neuron development by an unknown mechanism. Herein, we investigate how cAMP signaling promotes SA cell development by examining the role of CREB and PKA activation in development of NC cells to the SA lineage.

Transcription factors required for SA cell development include ASH1 or MASH1 (23–27), the mammalian homologue of Aschate-scute in Drosophila (28), and the paired homeobox transcription factors Phox2 (29), i.e., Phox2a (30) and Phox2b (31). In the developing embryo ASH1 is induced by BMPs (6) and precedes Phox2 and TH expression (32). ASH1+/− mice display defects in sympathetic ganglia (23) in catecholamine-producing cells of the adrenal medulla (25), and in Phox2a expression. Phox2a+/− mice lack the locus ceruleus, a major catecholaminergic center in the central nervous system (CNS), but Phox2b expression and sympathetic neuron development is largely normal (30). In Phox2b null embryos, NC cells migrate to the dorsal aorta and express MASH1, but
catecholaminergic neurons fail to develop (31). In vivo Phox2a or Phox2b overexpression generates additional noradrenergic and cholinergic neurons (33). By contrast, in vitro in primary NC cultures Phox2a overexpression induces TH expression and SA cell development only with activation of cAMP signaling (15). Thus, these studies (15) underscore the importance of the cAMP pathway in Phox2a action and SA cell development. cAMP signaling is also required for Phox2a-mediated transcription from the TH- and DBH-luciferase reporters studied in NC-derived PC12 cells and CNS-derived catecholaminergic Cath.a cells (34–36). Thus, cAMP signaling is important in Phox2a activation, studied by employing Phox2a-responsive reporter constructs in established cell lines. However, the mechanism by which cAMP signaling induces development of primary NC cells to the SA lineage and, specifically, the role of cAMP signaling in Phox2a regulation in NC cells is unknown.

Herein, we examine the role of the cAMP pathway in catecholaminergic cell development employing avian primary NC cultures, in conjunction with the CNS-derived catecholaminergic Cath.a cell line (37). This cell line provides another cellular model for comparative studies of NC-derived and CNS-derived catecholaminergic neuron development. The Cath.a cell line originated from brain tumors of transgenic mice expressing the SV40 T-antigen under control of the TH promoter (37). Cath.a cells, like catecholaminergic SA cells, are characterized by expression of TH and DBH, synthesis of catecholamines, and development of neurites. The CAD cell line used herein, a variant of the Cath.a cell line, undergoes neuronal differentiation characterized by expression of TH and development of neurites (17, 38), in response to serum withdrawal (38) or signals activating the cAMP pathway (17).

Herein, we demonstrate the molecular mechanism and the essential role of the cAMP pathway in catecholaminergic, sympathoadrenal lineage development; the cAMP pathway has dual inputs in sympathoadrenal cell development, namely, transcriptional induction of proneural Phox2a by CREB and regulation of Phox2a activity by PKA.

MATERIALS AND METHODS

Neural Crest Cell Cultures—Primary cultures of NC cells were prepared from I, quail embryos, stage 14–15 (39), as described (16). Briefly, 48-h embryo explants from trunk region of neural tube were cultured for 42 h. After removal of neural tube (Fig. 1A, day 0), the remaining cells are NC cells, assessed by HNK1-positive immunostaining. Mass cultures of NC cells (day 0) were harvested by trypsin treatment and replated at a density of 320 cells/mm2 (16). Clonal cultures of NC cells (day 0) were replated at density of 300 cells/35-mm dish in dishes coated with Vitrogen 100 (Collagen Corp.) and bovine fibronectin (20 μg/ml).

Growth Media and Other Reagents—Reagents for NC cultures were described in Bilodeau et al. (16). Concentrations of other reagents used were 10 ng/ml BMP2 (Wyeth Pharmaceuticals Inc.), 100 μM 3-isobutylmethylxanthine (IBMX; Sigma), 1–10 nm okadacid acid (OA; Sigma), and 5–10 μM H89 (Sigma), as indicated. Chicken embryo fibroblast cultures were from embryonic day 10 chicks, cultured as described (40, 41). CAD cells (38) were grown in Dulbecco’s modified Eagle’s medium (low glucose) containing 10% fetal bovine serum and 5–10% fetal calf serum. After the 2-h treatment, cells were assayed for luciferase activity. Luciferase activity was normalized per μg of protein extract.

Electrophoretic mobility shift assays were performed as described (42) employing 20 μg of nuclear extract (42) isolated from CAD cells treated with BMP2 and IBMX (24 h) followed by treatment (6 h) with 10 nM OA. 32P-Radiolabeled oligonucleotide probes containing the Phox2a cis-acting elements of DBH promoter were described in Adachi and Lewis (36).

Immunodetection and Histofluorescence—TH expression was monitored by Western blot analyses and immunofluorescence microscopy (16). Immunodetection of foreign protein expression, those fused to FLAG epitope, was via the M2 antibody (Sigma) or to E1A protein antibody (Lab Vision, Inc.). For immunofluorescence of Phox2a, CAD cells were permeabilized (30 min) in PBT (0.2% bovine serum albumin and 0.1% Triton X-100 in calcium-magnesium-free-phosphate-buffered saline (pH 7.4)) containing 1% goat serum. Phox2a antibody (Chemicon International) was added (1:200) in PBT containing 1% goat serum. Phox2a immunoprecipitations were performed with Phox2a antibody (Sigma) and 1 mg of whole cell extract (WCE) from CAD cells grown with IBMX, IBMX and OA (10 nM), or PD 98059 (20 μM). Phox2a immunoprecipitates were immunoblotted with 1:2000 and phosphoryl-Thr/ Ser-Pro-specific antibody (Cell Signaling). Western blot analyses for activated CREB employed phospho-CREB or CREB antibodies (Upstate) and WCE from NC cultures serum-starved (1 h) followed by treatment (10 min) with IBMX or forskolin (0.1–10 μM). Histofluorescence of catecholamines was performed as described in Furness et al. (43) and Bilodeau et al. (16).

RCASBP Vector Construction and Virus Preparation—ACREB (44), E1A (45, 46) and Δ2–32 E1A (47) were cloned into the Clal site of the RCASBP (A) vector by subcloning the PCR-generated inserts into SLAX12NCO plasmid (48). The CREBΔNHEJ-FLAG insert generated by “sticky-end PCR” (49) resulting in cohesive Clal ends, was subcloned in SLAX12NCO. All subcloned inserts were sequenced. RCAS BP (A) vectors were transfected in chicken embryo fibroblast cells by the calcium phosphate method. 7–10 days following transfection, supernatants were transfected and concentrated by ultracentrifugation (48, 50). Viral titers were determined by immunostaining for p19gag protein employing the ABC kit (Vector Laboratories). Typical titers were 109 IU/ml. NC cultures were infected at day 0 with 108 IU per 105 cells.

Real-time PCR—Total RNA was isolated by the Trizol method (Invitrogen) from NC cultures (grown in 35-mm dishes) or CAD cells. cDNA (20 μl) was synthesized from 2 μg of RNA. cDNA (2 μl) was used in quantitative real-time PCR as described in detail in Lee et al. (51).

RESULTS
cAMP Signaling in Synergy with BMP2 Regulates Phox2a mRNA Transcription—Primary murine (26) and avian (16) NC cultures require moderate activation of the cAMP pathway in synergy with BMP2 for SA lineage development. However, the mechanism by which cAMP signaling promotes SA cell development is unknown. To determine whether the cAMP pathway participates in SA cell development by regulating cAMP-responsive, CREB-mediated transcription, we expressed in primary NC cultures a dominant negative (44) or a constitutively active (52) variant of CREB, employing avian RCAS retroviral vectors (48). RCAS vectors were also used encoding the wild type (WT) Ad2 12 S E1A, which interferes with CBP function required for CREB-mediated transcription. The inactive N-terminal deletion Δ2–32 E1A was used as negative control (45, 46).

To establish the infection kinetics of the RCAS viruses and the expression of the foreign proteins, a time course was performed employing NC cultures infected at day 0 (Fig. 1A). Western blot analyses...
of cellular extracts isolated from virus-infected NC cultures demonstrate the expression of the foreign proteins (Fig. 1B). Immunofluorescence microscopy at 24, 48 (Fig. 1C), and 72 h (data not shown) post-infection demonstrates that nearly 100% of the cells express the foreign proteins.

Moderate activation of cAMP signaling by treatment with the cAMP-elevating agents IBMX (100 μM) or forskolin (0.1–1.0 μM) synergizes with BMP2, inducing expression of Phox2a (16). Herein, employing quantitative real-time PCR, we quantified Phox2a expression after treatment with IBMX in combination with BMP2. In comparison to IBMX, co-treatment with BMP2 and IBMX mediated a synergistic more than additive induction in Phox2a mRNA expression (Fig. 2A), suggesting the cAMP pathway together with BMP2 is involved in Phox2a transcription. Importantly, NC cultures treated with either IBMX or a low concentration of forskolin (0.1–1.0 μM) display CREB phosphorylation required for cAMP-mediated transcription (Fig. 2A).

To demonstrate the transcriptional involvement of the cAMP pathway in Phox2a expression, we examined the effects of dominant negative ACREB (44) and constitutively active CREB<sub>DIEDML</sub> (52) via infection of NC cultures with the respective RCAS viruses. Infection of NC cultures at day 0 (Fig. 1A) with the control RCAS virus in the presence of both BMP2 and IBMX results in a progressive increase of Phox2a mRNA at day 2, reaching a maximal level by day 3 (Fig. 2B). By comparison, infection with RCAS-ACREB virus in the presence of BMP2 and IBMX interferes with Phox2a mRNA induction (Fig. 2C), comparable with that with BMP2 and IBMX (Fig. 2B). Because CREB<sub>DIEDML</sub> is transcriptionally active (52) without activation of the cAMP pathway, these results (Fig. 2C) directly demonstrate the transcriptional involvement of the cAMP pathway in Phox2a mRNA expression via active CREB.

The co-activator CBP is required for CREB-mediated transcription (53). WT E1A represses CREB-dependent CBP function via direct interaction with CBP, whereas Δ<sup>2–32</sup> E1A does not interact with CBP and does not interfere with CBP function (45, 46). Accordingly, we examined the effect of WT E1A and its inactive Δ<sup>2–32</sup> mutant on Phox2a mRNA expression in NC cultures infected with the respective viruses. WT E1A reduces Phox2a mRNA by day 3, reaching a 50% reduction by day 4. By contrast, the Δ<sup>2–32</sup> E1A mutant has no noticeable effect (Fig. 2D). Although the mechanism of this E1A-mediated inhibition of

![Figure 1](image-url)
Phox2a mRNA expression has not been further investigated, the results support CBP involvement in Phox2a expression.

ACREB and WT E1A Repress the SA Phenotype—SA cells express TH, the rate-limiting enzyme in catecholamine biosynthesis, and DBH, the last enzyme required for the synthesis of catecholamines (CA). Thus, detection of the synthesized and stored catecholamines by histofluorescence (43) constitutes a definitive marker of SA cell development.

NC cultures infected with RCAS-ACREB, RCAS-WT E1A, and RCAS-Δ2–32 E1A viruses were examined for the appearance of the SA phenotype at day 6 of NC culture (Fig. 1A). SA cell markers assayed include both TH expression monitored by immunofluorescence microscopy and catecholamine biosynthesis by histofluorescence. ACREB or WT E1A interfered with the appearance of both TH-immunoreactive and catecholamine-positive cells in response to BMP2 and IBMX (Fig. 3A). By contrast, the control virus or expression of the inactive Δ2–32 E1A had no effect (Fig. 3A). Similar to Fig. 1C, parallel immunostaining experiments employing the M2 FLAG antibody, or the E1A antibody confirmed expression of these foreign proteins (data not shown). Because ACREB and WT E1A interfere with CREB and CBP, respectively, their inhibitory effect on the appearance of TH-immunoreactive and catecholamine-positive cells supports the direct transcriptional involvement of the cAMP pathway in SA cell development.
CREB\(_{DIEDML}\) Is Insufficient for SA Lineage Development—NC cultures infected with the RCAS-CREB\(_{DIEDML}\) virus in the presence of BMP2 alone did not increase the appearance of TH- and catecholamine-positive cells relative to cultures infected with control RCAS virus (Fig. 3B). This result was surprising because the expression of CREB\(_{DIEDML}\) with BMP2 did increase Phox2a mRNA expression (Fig. 2C). Interestingly, in CREB\(_{DIEDML}\)-expressing cultures, IBMX and BMP2 enhanced the appearance of both TH- and catecholamine-positive cells (Fig. 3B). Conversely, inhibition of PKA by the addition of the PKA inhibitor H89 (10\(\mu\)M) reversed the induction of the SA phenotype (Fig. 3B). Because increased Phox2a expression in the absence of PKA activation is insufficient for SA lineage development (Figs. 2C and 3B), we conclude the 1) CREB is necessary but insufficient in SA cell development and 2) cAMP signaling via PKA activation regulates additional events necessary for SA cell development.

To confirm these results we quantified TH protein expression by Western blot assays (16, 54). Consistent with our earlier observations (16), BMP2 and IBMX co-treatment of NC cultures infected with control RCAS virus resulted in a 2.6-fold increase in TH protein (Fig. 4, compare lanes 1, 3, and 4 to lane 2). ACREB in the presence of BMP2 and IBMX represses TH protein expression by 23% (compare lane 2 to lane 5). Interestingly, CREB\(_{DIEDML}\) in the presence of BMP2 and IBMX results in a 50% increase in TH protein expression (compare lane 2 to lane 6). By contrast, in the absence of IBMX, i.e., without PKA activation, CREB\(_{DIEDML}\) mediates only minimal TH protein expression (compare lane 2 to lane 10), which is similar to the BMP2-only control (lane 3). WT E1A (lane 7) decreased TH protein in comparison to the control (lane 2), whereas the inactive E1A\(_{\Delta32-32}\) had no effect (compare lane 2 to lane 8).

CREB and PKA Activation Are Necessary and Instructive in SA Lineage Development—The effect of microenvironmental signals on NC cell differentiation is either selective, by influencing cell proliferation and survival of a committed progenitor, or instructive, restricting the pluripotent nature of NC cells. Our earlier studies in NC cells (16) support that the influence of cAMP signaling on SA cell development is instruc-
A Ser/Thr Phosphatase Is Required for SA Cell Development—To further understand the mechanism by which activated PKA regulates SA lineage development, we based our investigations on the studies by Adachi and Lewis (36). In PC12 cells Phox2a is constitutively phosphorylated; the cAMP pathway promotes Phox2a dephosphorylation, increased DNA binding, and DBH-luciferase reporter expression (36). In CAD cells cAMP signaling regulates Phox2a-dependent DBH-luciferase reporter expression, which is inhibited by OA, an inhibitor of Ser/Thr phosphatases (36). Because in NC cells Phox2a is required for SA lineage development, we investigated whether the cAMP pathway regulates Phox2a activation via the same mechanism. Accordingly, NC cultures were induced to the SA lineage by BMP2 alone increased Phox2a mRNA expression (Fig. 2C). OA added for 3 h on day 0 of secondary NC culture decreased TH protein expression by 50% (Fig. 5B, compare lane 2 to lane 3). The addition of 10 nM OA for 3 h on day 1 of secondary NC culture mediates a smaller but reproducible decrease in TH expression (Fig. 5B, compare lane 5 to lane 6). This effect of OA on day 1 becomes more pronounced with increased duration (24 h) of treatment (Fig. 5C, compare lane 1 to lane 4). Furthermore, 24 h of OA treatment on day 1 reduced TH mRNA by ~30% without a significant effect on Phox2a mRNA (data not shown).

Because the inhibitory OA effect on TH expression and SA cell development occurs between 4 and 48 h of secondary NC culture (Fig. 5B and C), we interpret this result to mean the OA effect is specific, targeting the activity of early acting SA lineage-promoting transcription factor(s). Based on these results (Fig. 5) and those of Adachi and Lewis (36), we reasoned Phox2a is a likely target of the cAMP-mediated, OA-sensitive dephosphorylation required for SA cell development.

A Ser/Thr Phosphatase Is Required for Neuronal Differentiation of the CNS-derived CAD Cell Line—To confirm the role of the OA-sensitive Ser/Thr phosphatase in SA cell development via regulation of Phox2a activity, we employed the CAD cell line (37). CAD cells undergo neuronal differentiation characterized by expression of TH and development...
of neurites in response to serum withdrawal (38) or to cAMP-elevating agents (17). Treatment of CAD cells with BMP2 and IBMX increases CAD cell neuronal differentiation, measured by TH and peripherin immunoreactivity (Fig. 6A). Interestingly, 1 nM OA or 5 nM (data not shown) OA repressed both TH and peripherin immunoreactivity with- out an affect on Phox2a immunoreactivity (Fig. 6A). To confirm these results, we quantified by real-time PCR Phox2a and TH mRNA expres- sion in CAD cells induced to differentiate by the addition of BMP2 and IBMX as a function of OA (1 nM). BMP2 and IBMX induce transcription of both TH and Phox2a mRNAs. By contrast, 1 nM OA inhibits TH mRNA expression (Fig. 6B) without a significant effect on Phox2a expression (Fig. 6C). These results are also supported by the immuno- fluorescence data of CAD cells (Fig. 6A), demonstrating inhibition of TH and peripherin immunostaining in the presence of OA without an affect on Phox2a immunostaining. These observations suggest a regu- latory role for the OA-sensitive PP2A-like phosphatase, regulating the activity of Phox2a, which in turn mediates TH expression and neurite outgrowth.

Amino acid sequence alignment of Phox2a proteins identified puta- tive, evolutionarily conserved protein kinase phosphorylation sites. These include phosphorylation sites for glycogen synthase kinase 3, extracellular signal-regulated kinase 1/2, PKA, calmodulin kinase II, and protein kinase C (Fig. 7A). Although more detailed studies are required to demonstrate the functional significance of these putative phospho- rylation sites in Phox2a, the evolutionary conservation of functionally important regulatory sequences is a well accepted biological phenome- non. Thus, we hypothesized that these conserved Phox2a phosphoryl- ation sites are of regulatory significance and are potential targets of dephosphorylation by cAMP-mediated activation of a PP2A-like phosphatase.

To directly demonstrate that Phox2a phosphorylation is regulated by cAMP signaling and the OA-sensitive PP2A-like phosphatase, we examined Phox2a immunoprecipitates for the presence of phosphorylations at Ser/Thr residues that are likely phosphorylation sites of proline-directed kinases, such as mitogen-activated kinases. Phox2a immu-noprecipitates derived from CAD cells grown under control conditions or after treatment with IBMX, IBMX and OA (10 nM), or PD 98059 were immunoblotted with a phospho-Thr/Ser-Pro-specific antibody (Fig. 7B). The Phox2a phosphorylation status demonstrates a statistically significant reduction ($p < 0.05$) after treatment with IBMX, and impor-
stantly, this reduction is reversed by OA. Likewise, treatment with PD 98059, a specific inhibitor of MEK1, also reduces Phox2a phosphorylation, confirming the specificity of the phospho-Thr/Ser-Pro-specific antibody and suggesting these phosphorylations include putative S-P phosphorylation sites (Fig. 7A). These results (Fig. 7B) demonstrate that cAMP signaling modulates via an OA-sensitive phosphatase the phosphorylation of Phox2a.

**Phox2a Dephosphorylation Induces Phox2a DNA Binding in Vitro and Phox2a Transactivation**—To determine the functional importance of the cAMP-dependent and OA-sensitive dephosphorylation of Phox2a, we initially determined the interval of OA sensitivity, abrogating CAD cell neuronal differentiation. As shown in Fig. 8A, the addition of 5 nM OA for 12–18 h after 24 h of treatment with BMP2 and IBMX inhibits neuronal differentiation of CAD cells, assessed by immunofluorescence microscopy for peripherin expression. Our current studies have defined that 24 h of BMP2 and IBMX treatment of CAD cells is necessary for cell cycle exit and differentiation. Accordingly, CAD cells were treated with BMP2 and IBMX for 24 h followed by 6 h of treatment with OA. CAD cell nuclear extracts were isolated at 6 h ± OA treatment and utilized in electrophoretic mobility shift assays employing the Phox2a cis-acting elements of the DBH promoter (36). A sequence-specific Phox2a-DNA complex is detected in extracts isolated from CAD cells treated with BMP2 and IBMX, based on competition assays with a 30-fold excess of unlabeled WT versus mutant oligonucleotides (Fig. 8B). This sequence-specific complex is supershifted with the Phox2a antibody but not with IgG, thus confirming the identity of the Phox2a-DNA complex (Fig. 8B). Importantly, this Phox2a-DNA complex is absent or reduced in extracts isolated from control, untreated CAD cells or extracts isolated from CAD cells grown in the presence of OA (Fig. 8B). These observations support that cAMP-dependent Phox2a dephosphorylation is required for Phox2a DNA binding, in agreement with earlier studies (36).

To directly link the functional importance of the OA effect on Phox2a activity, a Phox2a expression vector (36) was transiently co-transfected in CAD and NC cells with the DBH-luciferase reporter as a function of BMP2 and IBMX treatment with and without OA addition (Fig. 8C). In CAD cells (data not shown) and NC cells (Fig. 8C), OA suppresses BMP2- and IBMX-mediated expression from the Phox2a-dependent

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* M. Paris and O. Andrisani, manuscript in preparation.
DBH-luciferase reporter, thus directly linking the OA effect on Phox2a transcriptional activity. Taken together, the results demonstrate that OA inhibits cAMP-dependent Phox2a dephosphorylation (Fig. 7B), cAMP-dependent Phox2a DNA binding (Fig. 8B), cAMP-dependent Phox2a transactivation (Fig. 8C), and cAMP-dependent NC (Fig. 5) and CAD cell differentiation (Fig. 8A).

**DISCUSSION**

In this study we investigated the molecular mechanism by which the cAMP pathway induces SA lineage development, employing primary cultures of NC cells. We demonstrate CREB activation is necessary in synergy with BMP2 to induce Phox2a transcription. Furthermore, PKA activation via a PP2A-like phosphatase is required for activation of the transcriptional activity of Phox2a and development of the SA lineage. Because transcription mediated by Phox2a is necessary for SA cell development (29–31), this PKA-dependent transcriptional activation of Phox2a leading to SA lineage development establishes for the first time that moderate activation of cAMP signaling is an essential, instructive signal in SA lineage development.

**ACREB Inhibits Phox2a Transcription and SA Cell Development**—In NC cultures, ACREB suppresses Phox2a expression and the appearance of TH- and catecholamine-positive cells without affecting NC cell survival. Accordingly, we conclude cAMP-dependent transcription mediated by CREB is necessary in instructing NC cells to the SA lineage. This conclusion agrees with the phenotype of CREB−/− mice (22), which displays a 65% reduction in superior cervical ganglia sympathetic neuron development. This 65% reduction, as opposed to 100%, which...
occurs earlier than the CREB-dependent, neurotrophin-mediated survival and growth (22), is probably due to the redundancy of CREB/ATF family of proteins. Importantly, our studies extend these observations by identifying CREB as necessary in Phox2a transcription.

The direct involvement of CREB in transcription from the Phox2a promoter in synergy with BMP2 is supported by our recent studies (58). A cluster of functional CRE half-sites have been identified in proximity to E-box DNA binding sites that are potential binding sites for ASH1. These CRE and E-box sites are evolutionarily conserved in mouse and human Phox2a promoters. Importantly, the Phox2a gene has been identified by the serial analysis of chromatin occupancy (SACO) method (59) as a CREB-regulated gene, in agreement with our findings (58).

**CREBDIEDML Induces Phox2a mRNA but Not SA Cell Development**

In NC cultures, CREBDIEDML in the presence of BMP2 increased Phox2a mRNA expression, thus, directly demonstrating that activated CREB is necessary for Phox2a mRNA expression. Interestingly, CREBDIEDML expressing NC cells, despite the increased Phox2a expression, do not develop to the SA lineage unless the cAMP pathway is activated, in agreement with the Phox2a overexpression studies of Lo et al. (15). Because Phox2a transcription induced by CREBDIEDML is insufficient for SA lineage development unless PKA is activated, our studies establish the PKA dependence in the development of the SA lineage.

**Involvement of a Ser/Thr Phosphatase in SA Lineage Development**

In PC12 and Cath.a cells, Phox2a activation, studied via the DBH-driven reporter (36), requires activation of the cAMP pathway and is inhibited by OA. In this study employing pluripotent NC cells, Phox2a activation studied in the context of SA lineage development requires the cAMP pathway mediating activation of an OA-sensitive Ser/Thr phosphatase. This OA-sensitive event does not affect NC cell survival and occurs early in NC cell development, when proneural Phox2a initiates development of the SA lineage. Because low concentrations of OA inhibit the phosphatase 2A family of proteins (55, 56), we propose PP2A or a PP2A-like phosphatase (56) regulates SA lineage specification by dephosphorylating Phox2a.

Interestingly, during retina development in *Xenopus*, the activity of NeuroD is positively regulated by dephosphorylation at its glycogen synthase kinase 3 phosphorylation site (60). Furthermore, in *Drosophila*, PP2A in association with the B' subunit is essential in positively regulating the activity of the homeodomain protein SCR (61). In addition, PP2A in complex with the B56α subunit positively regulates the activity of the basic helix-loop-helix transcription factors HAND1 and HAND2 involved in heart, vascular, neuronal, and extraembryonic development (62). Thus, the precedent exists for a role of PP2A in pos-
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In summary, we demonstrate the essential, instructive role of the cAMP pathway in SA cell development (Fig. 9). The transcriptional components of the cAMP pathway, CREB and CBP, induce Phox2a transcription acting instructively in SA cell specification. In addition, the cAMP-dependent PKA regulates the activity of a Ser/Thr PP2A-like phosphatase involved in Phox2a activation. Thus, the cAMP pathway mediates a multilevel control in SA cell development, regulating both Phox2a transcription and activity. We conclude the cAMP pathway is necessary and instructive in SA cell specification.

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REFERENCES

1. Le Douarin, N. M., Creuzet, S., Couly, G., and Dupin, E. (2004) Development 131, 4637–4650
2. LaRonne, C., and Bronner-Fraser, M. (1998) J. Neurobiol. 36, 175–189
3. Anderson, D. J., Carnahan, J. F., Michelsohn, A., and Patterson, P. H. (1991) J. Neurosci. 11, 3507–3519
4. Reisman, E., Ernsberger, U., Francis-West, P. H., Rueger, D., Brickell, P. D., and Rohrer, H. (1996) Development 122, 2079–2088
5. Shah, N. M., Groves, A., and Anderson, D. J. (1996) Cell 85, 331–343
6. Schneider, C., Wicht, H., Enderich, J., Wegner, M., and Rohrer, H. (1999) Neuron 24, 861–879
7. Varley, J. E., Webb, R. G., Rueger, D. C., and Maxwell, G. D. (1995) Dev. Dyn. 203, 434–447
8. Varley, J. E., and Maxwell, G. D. (1996) Dev. Neurosci. 18, 84–94
9. Varley, J. E., McPherson, C. E., Zou, H., Niswander, L., and Maxwell, G. D. (1998) Dev. Biol. 196, 107–118
10. Cohen, A. M. (1972) J. Exp. Zool. 179, 167–182
11. Le Douarin, N. M., Teillet, M. A., and Le Lièvre, C. (1977) Soc. Gen. Physiol. Ser. 32, 11–27
12. Groves, A. K., George, K. M., Tissier-Seta, J. P., Engel, J. D., Brunet, J. F., and Anderson, D. J. (1995) Development 121, 887–901
13. Stern, C. D., Artinger, K. B., and Bronner-Fraser, M. (1991) Development 113, 207–216
14. Teillet, M. A., and Le Douarin, N. M. (1983) Dev. Biol. 98, 192–211
15. Lo, L., Morin, X., Brunet, J. F., and Anderson, D. J. (1999) Neuron 22, 693–705
16. Bilodeau, M. L., Bouliveau, T., Hullinger, R., and Andrisani, O. M. (2000) Mol. Cell. Biol. 20, 3004–3014
17. Bilodeau, M. L., Ji, M., Paris, M., and Andrisani, O. M. (2005) Mol. Cell. Neurosci. 29, 394–404
18. Pierce, K. L., Premont, R. T., and Lefkowitz, R. J. (2002) Nat. Rev. Mol. Cell Biol. 3, 639–650
19. Andrisani, O. M. (1999) Crit. Rev. Eukaryot. Gene Expr. 9, 19–32
20. Montminy, M. (1997) Annu. Rev. Biochem. 66, 807–822
21. Goodman, R. H., and Smolik, S. (2000) Genes Dev. 14, 1553–1577
22. Lonze, B. E., Riccio, A., Cohen, S., and Ginty, D. (2002) Neuron 34, 371–385
23. Guillemot, F., Lo, L.-C., Johnson, J. E., Auerbach, A., Anderson, D. J., and Joyner, A. L. (1993) Cell 75, 463–476
24. Hirsh, M. B., Tiveron, M. C., Guillemot, F., Brunet, J. F., and Goridis, C. (1998) Development 125, 599–608
25. Huber, K., Brühl, B., Guillemot, F., Olson, E. N., Ernsberger, U., and Unserick, K. (2002) Development 129, 4729–4738
26. Lo, L., Sommer, L., and Anderson, D. J. (1997) Curr. Biol. 7, 440–450
27. Sommer, L., Shah, N., Rao, M., and Anderson, D. J. (1995) Neuron 15, 1245–1258
28. Johnson, J. E., Birren, S. J., and Anderson, D. J. (1990) Nature 346, 858–861
29. Brunet, J. F., and Pattyn, A. (2002) Curr. Opin. Genet. Dev. 12, 435–440
30. Morin, X., Cremer, H., Hirsch, M.-R., Kapur, R. P., Goridis, C., and Brunet, J.-F. (1997) Neuron 18, 411–423
31. Pattyn, A., Morin, X., Cremer, H., Goridis, C., and Brunet, J.-F. (1998) Nature 399, 366–370
32. Ernsberger, U., Patake, H., Tissier-Seta, J. P., Reh, T., Goridis, C., and Rohrer, H. (1995) Mech. Dev. 52, 125–136
33. Stanke, M., Jungbauer, D., Geissen, M., Goridis, C., Ernsberger, U., and Rohrer, H. (1999) Development 126, 4087–4094
34. Zellmer, E., Zhang, Z., Greco, D., Rhodes, J., Cassel, S., and Lewis, E. J. (1995) J. Neurosci. 15, 8109–8120
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35. Swanson, D. J., Zellmer, E., and Lewis, E. J. (1997) J. Biol. Chem. 272, 27382–27392
36. Adachi, M., and Lewis, E. (2002) J. Biol. Chem. 277, 22915–22924
37. Suri, C., Fung, B. P., Tischler, A. S., and Chikaraishi, D. M. (1993) J. Neurosci. 13, 1280–1291
38. Qi, Y., Wang, J. K., McMillian, M., and Chikaraishi, D. M. (1997) J. Biol. Chem. 272, 27382–27392
39. Hamburger, V., and Hamilton, H. L. (1992) Dev. Dyn. 195, 231–272
40. Fekete, D. M., and Cepko, C. L. (1993) Mol. Cell. Biol. 13, 2604–2613
41. Hunter, E. (1979) Methods Enzymol. 58, 379–393
42. Andrisani, O. M., Pot, D. A., Zhu, Z. and Dixon, J. E. (1988) Mol. Cell. Biol. 8, 1947–1956
43. Furness, J. B., Costa, M., and Wilson, A. J. (1977) Histochemistry 52, 159–170
44. Ahn, T., Olive, M., Aggarwal, S., Krylov, D., Ginty, D. D., and Vinson, C. (1998) Mol. Cell. Biol. 18, 967–977
45. Arany, Z., Newsome, D., Oldread, E., Livingston, D. M., and Eckner, R. (1995) Nature 374, 81–84
46. Lundblad, J. R., Kwok, R. P. S., Laurance, M. E., Harter, M. L., and Goodman, R. H. (1995) Nature 374, 85–88
47. Stein, R. W., Corrigan, M., Yaciuk, P., Whelan, J., and Moran, E. (1999) J. Virol. 64, 4421–4427
48. Morgan, B. A., and Fekete, D. M. (1996) Methods Cell Biol. 51, 185–218
49. Zeng, G. (1998) Biotechniques 25, 206–208
50. Hollenbeck, P. J., and Fekete, D. M. (2003) Methods Cell Biol. 71, 369–386
51. Lee, S., Tarn, C., Wang, W.-H., Chen, S., Hullinger, R., and Andrisani, O. (1999) J. Biol. Chem. 274, 8730–8740
52. Cardinaux, J. R., Notis, J. C., Zhang, Q. H., Vo, N., Craig, J. C., Fass, D. M., Brennan, R. G., and Goodman, R. H. (2000) Mol. Cell. Biol. 20, 1546–1552
53. Chrvicha, J. C., Kwok, R. P. S., Lamb, N., Hagiwara, M., Montminy, M. R., and Goodman, R. H. (1993) Nature 365, 855–859
54. Bilodeau, M. L., Boulineau, T., Greulich, J. D., Hullinger, R., and Andrisani, O. M. (2001) In Vitro Cell. Dev. Biol. Anim. 37, 185–192
55. Cohen, P., Khumpp, S., and Schilling, D. L. (1989) FEBS Lett. 250, 596–600
56. Cohen, P. (1997) Trends Biochem. Sci. 22, 245–251
57. Ji, M. and Andrisani, O. M. (2005) Mol. Cell. Biol. 25, 5134–5145
58. Benjamin, C., Paris, M., Wang, W.-H., Hong, S. J., Kim, K. S., Hullinger, R. L., and Andrisani, O. M. (2005) 64th Annual Meeting of Society of Developmental Biology, San Francisco, July 27–August 1, 2005, Abstract 143, Society of Developmental Biology
59. Impney, S., McCormick, S. R., Cha-Molstad, H., Dwyer, J. M., Yochum, G. S., Ross, J. M., McWeeney, S., Dunn, J. J., Mandel, G., and Goodman, R. H. (2004) Cell 119, 1041–1054
60. Moore, K. B., Schneider, M. L., and Vetter, M. L. (2002) Neuron 34, 183–195
61. Berry, M. and Gehring, W. (2000) EMBO J. 19, 2946–2957
62. Firulli, A. B. (2003) Mol. Cell 12, 1225–1237