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The Phox2 homeodomain proteins are sufficient to promote the development of sympathetic neurons

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SUMMARY

The development of sympathetic neurons is controlled by a network of transcriptional regulators, including the paired homeodomain proteins Phox2a and Phox2b. To understand the role of Phox2 proteins in more detail, the effect of Phox2 overexpression was analysed in the avian peripheral nervous system. Phox2a expression in neural crest cultures elicited a strong increase in the number of sympathoadrenergic cells. Expression of Phox2a in the chick embryo promoted the generation of additional neurons expressing the noradrenergic marker genes *DBH* and *TH*, pan-neuronal genes *SCG10* and *NF160* and cholinergic genes *ChAT* and *VAChT*. Phox2a-induced neurons were found in ectopic locations such as dorsal root ganglia and peripheral nerve. Sympathoadrenergic development could be elicited in cultures of E5 dorsal root ganglia, demonstrating the presence of Phox2a-responsive cells in non-autonomic peripheral ganglia. Phox2b induced ectopic neurons in the chick embryo in the same way as Phox2a. These results show that Phox2 proteins are sufficient to promote sympathetic neuron generation and control, directly or indirectly, the expression of a large number of genes characteristic for sympathetic neurons.

Key words: Noradrenergic, Neural crest, Transmitter phenotype, Neuronal identity, *Cash1*, *Mash1*

INTRODUCTION

The function of the nervous system depends on the interaction of distinct classes of neurons generated during development at characteristic locations. The determination of neuronal identity appears to be controlled by extrinsic signals produced by organisation centers within the nervous system and by neighbouring non-neuronal tissues (Jessell and Lumsden, 1998; Ensini et al., 1998). These extrinsic signals seem to determine neuronal identity by directing the expression of a network of transcription factors. The signal transduction pathways leading from the activation of cell surface receptors to the expression of transcription factors and finally to the activation of characteristic terminal differentiation genes are still poorly understood. The observation that the expression of different aspects of neuronal identity can be experimentally uncoupled (Groves et al., 1995; Sommer et al., 1995) implies that different components of neuronal identity may be specified by distinct subprograms.

One important aspect of neuronal identity is the neurotransmitter phenotype, i.e. the set of genes responsible for the production, uptake and storage of a specific neurotransmitter. Work in both invertebrate and vertebrate nervous systems has identified transcriptional regulators controlling the development of GABAergic, serotonergic, dopaminergic and noradrenergic phenotypes (Goridis and Brunet, 1999). For the noradrenergic phenotype of the vertebrate nervous system, the bHLH transcription factor Mash1 (Guillemot et al., 1993; Hirsch et al., 1998) and the paired-homeodomain proteins Phox2a (Morin et al., 1997) and Phox2b (Pattyn et al., 1997, 1999) were shown to be essential. In Mash1 knockout mice, the noradrenaline-synthesizing enzyme dopamine β-hydroxylase (DBH) is absent in the CNS and in most noradrenergic cells of the PNS (Guillemot et al., 1993; Hirsch et al., 1998). The action of Mash1 may be mediated in part by Phox2a, which is a direct or indirect downstream target of Mash1 (Hirsch et al., 1998). Indeed, in Phox2a-deficient mice, the major noradrenergic center of the brain, the locus coeruleus, does not develop and its precursors never express DBH (Morin et al., 1997).

Mash1 and Phox2 proteins are also essential for the development of noradrenergic neurons of the PNS, involving a complex interaction between the Mash1-controlled *Phox2a* gene and *Phox2b*, whose expression does not depend on Mash1 (Hirsch et al., 1998; Pattyn et al., 1999). *Phox2* genes seem to directly regulate the expression of DBH, since both Phox2a and Phox2b bind to the DBH promotor and transactivate a DBH reporter gene (Zellmer et al., 1995; Yang et al., 1998; Kim et al., 1998). The *Phox2a* and *Phox2b* knockouts affect not only the expression of noradrenergic traits but, in several neuronal lineages, cells are either not generated or die at the neuron precursor stage. This suggests that other genes, involved in

neurogenesis and/or neuron survival, are also Phox2 target genes. There is considerable evidence to suggest that the gene encoding the neurotrophic factor receptor c-ret is regulated by Phox2 proteins (Morin et al., 1997; Lo et al., 1998; Pattyn et al., 1999). However, as only part of the lineages affected in Phox2-deficient animals depend on c-ret, other aspects of neuronal differentiation, including further neurotrophic factor receptors, may be controlled in addition by *Phox2* genes.

In the present study, we asked whether the expression of *Phox2a* and *Phox2b* genes is sufficient to direct noradrenergic differentiation of neural precursors. In addition, we asked what cells respond to Phox2 proteins and which genes are controlled by Phox2 proteins, besides the subprogram of genes characteristic for noradrenergic neurons. Using a retroviral vector to express *Phox2* genes in cultures of avian neural crest and dorsal root ganglia (DRG) and in the chick embryo in vivo, we observed that *Phox2* genes are sufficient to induce the generation of neurons that express pan-neuronal, noradrenergic and cholinergic genes. As these cells display a gene expression pattern characteristic for developing sympathetic ganglia and not found in enteric or parasympathetic ganglia, they are termed here sympathetic neurons.

MATERIALS AND METHODS

Neural crest and DRG cultures

Primary culture of quail trunk neural crest (NC) was performed using a modification of the protocol described by Fauquet et al. (1981). Neural tubes from 2-day-old quail embryos were dissected from the trunk region, carefully cleaned from adhering connective tissue by pancreatin treatment, plated on a collagen substratum and grown in DMEM with 15% CEE (chicken embryo extract) and 15% HS (horse serum). After 24 hours at 37°C and 10% CO_2, neural tubes were removed and the remaining migrating NC cells were detached with 0.1% trypsin in PBS. After stopping the reaction with trypsin inhibitor, pelleting by centrifugation and resuspending, 7000 cells were replated in wells (10 mm diameter) of collagen-coated dishes (Greiner). After 3 hours, when cells had attached, Phox2a-RCAS virus concentrate (final titer 7.5×10^7/ml) or control virus was added. Cells were grown for 5 days after replating and were then analysed by immunocytochemistry or in situ hybridisation. Cultures were maintained in the absence of bone morphogenetic proteins (BMPs).

DRGs were dissected from day 5 (E5) chick embryos, treated with 0.1% trypsin for 4 minutes, dissociated to single cells, plated onto collagen-coated wells at a density of 5×10^4 cells in neural crest culture medium described above and grown for 5 days. Phox2a-RCAS virus concentrate or control virus was added 3 hours after plating.

Immunocytochemistry on neural crest and DRG cultures

Staining for tyrosine hydroxylase (TH) was carried out using mouse anti-TH mAb (Rohrer et al., 1986), followed by Cy3-labeled donkey anti-mouse antibodies (Jackson Laboratories; through Dianova, Hamburg, Germany) as previously described (Ernsberger et al., 1989). In some cases, staining for TH was combined with staining for Phox2a, using monoclonal mouse anti-TH and rabbit anti-Phox2a antibody (Tiveron et al., 1996). To analyse the effect of Phox2a on TH expression, the whole area of the culture dish was analysed by counting the total number of adrenergic cells. The total number of neural crest and DRG cells was determined by counting the total number of cell nuclei in 10 randomly selected visual fields after staining of the nuclei with Hoechst dye. Data from at least three independent experiments are shown for the effects on TH-positive cells in neural crest and DRG cultures. Values are given as means ± s.e.m.

Retroviral constructs

For the construction of retroviral vectors, full-length mouse Phox2a and Phox2b cDNA was amplified using standard PCR protocols and *Cla*I restriction sites, flanking the coding sequence, were added. Furthermore a Kozak-like consensus sequence was introduced at the 5′ end. The sequence was inserted into RCASBP(B) viral cloning vector by using the *Cla*I restriction sites. The fragment was cloned in sense orientation into the RCAS(B) (Hughes and Kosik, 1984; Morgan and Fekete, 1996) and verified via sequence analysis (Phox2a-RCAS, Phox2b-RCAS). Control virus contained a fragment of CNTFRα (Heller et al., 1995) in antisense orientation. SPF chick embryo fibroblasts were transfected with the retroviral DNA and maintained until complete viral infection of the cultured cells, as revealed using an antibody against the viral protein gag (mAb AMV-3C2) or anti-Phox2a antibody (Tiveron et al., 1996). mAb AMV-3C2, developed by D. Boettiger, was obtained from the Developmental Studies Hybridoma Bank, University of Iowa, Iowa City.

Expression of Phox2 in vivo using Phox2-RCAS

Fertilized virus-free chicken eggs were obtained from Lohmann, Cuxhaven, Germany and incubated for 2 days. After opening the egg shell and staging the embryos, aggregates of infected Phox2a-RCAS, Phox2b-RCAS or control virus-producing chick embryo fibroblasts were implanted into the embryos at brachial levels between the neural tube and the last somite formed (Reissmann et al., 1996). The eggs were sealed with tape and incubated until E7-9. Embryos were killed by decapitation. Trunk and cervical region of the embryos were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.3) overnight, kept in 15% sucrose (in 0.1 M phosphate buffer, pH 7.3) overnight, embedded in Tissue-Tek (Sakura Finetek Europe BV, Zoeterwoude Netherlands) and sectioned. Consecutive 12 μm cryostat cross-sections were separately collected and analysed for expression of TH, DBH, SCG10, NF160, ChAT, VAChT, Phox2a, Phox2b, c-ret and viral reverse transcriptase (RT) by in situ hybridization. At least three embryos were analysed for each of the genes investigated.

In situ hybridisation of sections and cultured cells

Non-radioactive in situ hybridization on cryosections and cultured cells and preparation of digoxigenin-labeled probes for chick TH, ChAT and SCG10 were carried out using a modification of the protocol of D. Henrique (IRFDBU, Oxford, UK) as previously described (Ernsberger et al., 1997). The following cRNA probes were used in addition: the chick homologues of Phox2a (cPhox2a; Ernsberger et al., 1995), NF 160 (Zopf et al., 1987), c-ret (Robertson and Mason, 1995), Phox2b (cPhox2b), DBH, VAChT and RCAS RT. cPhox2b, a 357 bp DNA fragment corresponds to bases 278-635 of the mouse Phox2b sequence (Pattyn et al., 1997), a 580 bp cDBH fragment corresponding to bases 727-1207 of the human DBH sequence (U. E. et al., unpublished data), and cVACHT, a 800 bp DNA fragment corresponding to bases 1578-2071 of the human sequence and including in addition the 3′ untranslated region of cVACHT (M. S. et al., unpublished data), were amplified from RNA isolated from E8 chick sympathetic ganglia with degenerate oligonucleotide primers. The probe for RCAS-RT was made from a 405 bp *Bam*HI fragment, corresponding to bases 3301-3706 of RCASBP(B), subcloned into pBSK (S. Heller, personal communication).

RESULTS

To investigate the role of Phox2 proteins, quail neural crest cells were infected with a Phox2a-expressing RCAS virus (Phox2a-RCAS) and analysed for the number of sympathoadrenergic cells. Infection by Phox2a-RCAS at day 1 of the culture resulted in a dramatic increase in the number of TH-expressing cells (Fig. 1A). The total number of cells was

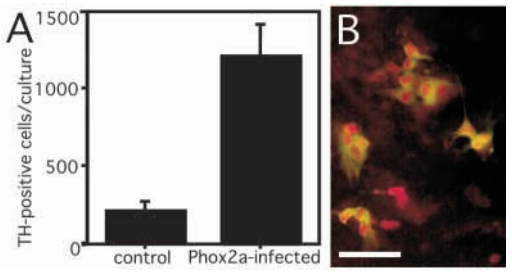

Fig. 1. Phox2a-expression in neural crest cells elicits an increase in the number of TH-positive cells. (A) The number of TH-positive cells in control and Phox2a-RCAS-infected cultures are quantified. Data represent the mean ± s.e.m. of 4 independent experiments. (B) Double-immunostaining for TH (green) and Phox2a (red) in Phox2a-RCAS-infected neural crest cells after 5 days of culture. Note that all TH-expressing cells coexpress Phox2a-IR. Magnification bar, 50 μm.

not significantly affected by Phox2a-RCAS infection (33.792±2.244 cells/culture in control as compared to 37.224±4.356 in Phox2a-RCAS-infected cultures; mean ± s.e.m.; *n*=3), excluding the possibility that the increase in TH-positive cells is reflecting a general increase in total cell number. All TH-expressing cells co-expressed Phox2a in the nucleus, as shown by double-staining for TH and Phox2a (Fig. 1B).

As neural crest precursor cells may reveal in culture a developmental potential not realized in their normal environment (Xue et al., 1985; Richardson and Sieber-Blum, 1993; Stocker et al., 1991; Pomeranz et al., 1993), it was important to analyse the effects of Phox2a overexpression also in vivo, in the developing chick embryo. Aggregates of Phox2a-RCAS-producing fibroblasts were implanted into the ventral neural crest migration pathway between the somites and the neural tube, in the vicinity of the dorsal aorta. This results in infection of neural crest, neural-crest-derived peripheral ganglia and peripheral nerves and neighbouring mesenchymal tissues (Fig. 2A; see also Reissmann et al., 1996). The infection was largely restricted to the side of implantation (Reissmann et al., 1996), but spread dorsal and ventral of the axial structures onto the contralateral side in some embryos. The effect of Phox2a overexpression was analysed at embryonic days 7-9 since at this age, the secondary paravertebral ganglia have formed and both adrenergic, cholinergic and pan-neuronal marker genes are expressed (Ernsberger et al., 1995, 1997; Vogel and Weston, 1990). The major effect of Phox2a overexpression consisted of an increase in the size of sympathetic ganglia (Fig. 2B) and in ectopically located TH-expressing cells, in particular cells present in the spinal (brachial) nerve and in the DRG (Fig. 2B,C). Cells with sympathoadrenergic properties were never observed in ectopic positions in normal embryos or embryos infected with unrelated control virus (not shown). In addition, the presence of ectopic neurons correlated with the infection by Phox2a-RCAS virus.

Since the cells in the peripheral nerve are most easily identified as ectopic, Phox2a-induced cells, the properties of these cells were documented in detail. Cells in the enlarged sympathetic ganglia and in the DRG display the same phenotype (not shown). The expression of Phox2a resulted in the generation of neurons in the brachial nerve, characterized by the expression of the (nor)adrenergic marker genes *TH* and *DBH* and of the pan-neuronal genes *SCG10* and *NF160* (Fig. 3). Similar numbers of cells expressing noradrenergic or pan-neuronal marker genes were observed on consecutive sections. Avian sympathetic ganglia contain, in addition to noradrenergic neurons, a considerable number of cholinergic neurons (Ernsberger et al., 1997). As the onset of expression of the cholinergic locus in sympathetic ganglia occurs at E6.5, we asked whether cholinergic properties are detected in ectopic neurons. Cells expressing VAChT and ChAT were observed in Phox2a-RCAS-infected nerves (Fig. 3), although in lower numbers as compared to noradrenergic neurons. The presence of cells expressing the cholinergic marker genes *ChAT* and *VAChT* in peripheral nerve (Fig. 3) demonstrates that Phox2a promotes the generation of neurons expressing pan-neuronal, noradrenergic and cholinergic genes, a gene expression pattern characteristic for embryonic chick sympathetic neurons. As Phox2a is able to induce sympathetic neuron development also in the DRG, an environment that is non-permissive for noradrenergic differentiation during normal chick development (Xue et al., 1985; Le Lievre et al., 1980), we conclude that Phox2a expression is sufficient to initiate sympathetic neuron generation.

The presence of ectopic noradrenergic neurons in DRG of Phox2a-RCAS-infected embryos (Fig. 2) indicates that Phox2a-responsive precursor cells are present in this tissue. To

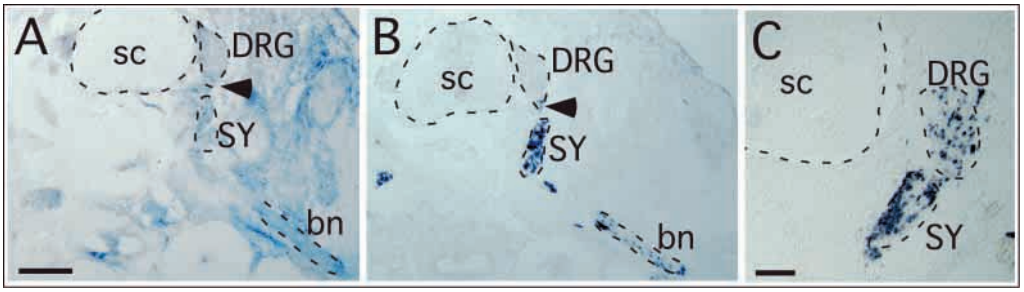

Fig. 2. Phox2a expression in the developing chick embryo results in increased size of sympathetic ganglia and ectopic TH mRNA-expressing cells in DRGs and peripheral nerves. (A) Infection of chick embryos with Phox2a-RCAS at brachial level results at E8 in largely unilateral infection of the peripheral nervous system (DRG, sympathetic ganglia, brachial nerve) and surrounding mesenchymal tissues, as revealed by in situ-hybridisation for viral reverse transcriptase (RT). Arrowhead indicates position of ectopic TH-positive cells in DRG (B). (B) On consecutive sections probed for TH by in situ hybridisation, enlarged sympathetic ganglia and ectopic TH-positive cells in the brachial nerve and DRG (arrowhead) were observed in the infected region. (C) At higher magnification, a different section from the infected region with a larger number of TH-positive cells in the DRG. Labelled features are outlined by dashed lines. sc, spinal cord; DRG, dorsal root ganglion; SY, sympathetic ganglion; bn, brachial nerve. Magnification bars, 500 μm and 200 μm in A-C, respectively.

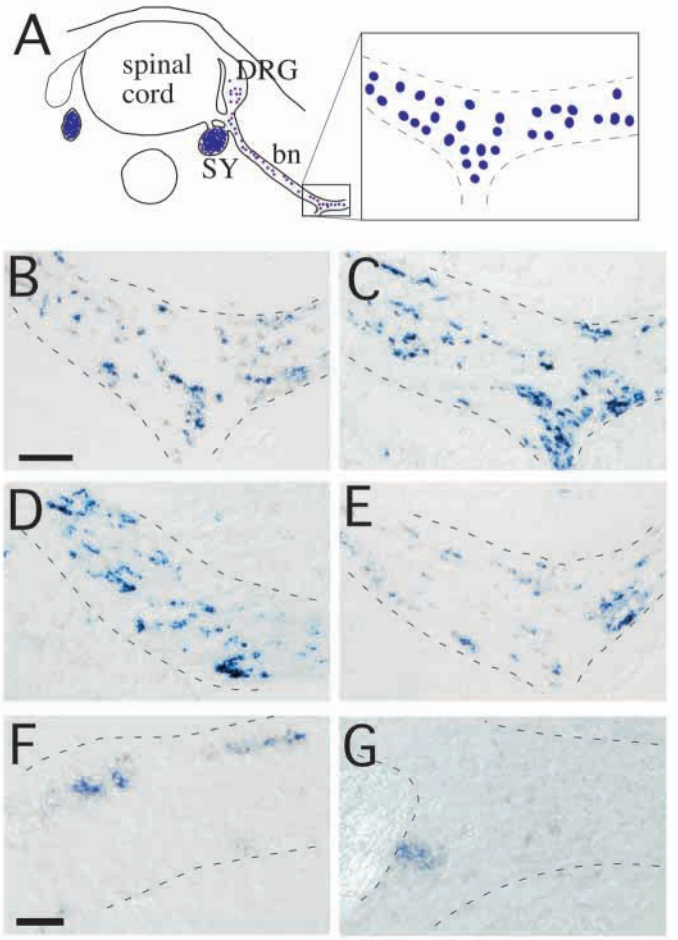

Fig. 3. Phox2a expression in the chick embryo results in ectopic cells in peripheral nerve, expressing noradrenergic, pan-neuronal and cholinergic marker genes. (A) The schematic drawing shows the distribution of ectopic cells in the brachial nerve and the area chosen for documentation, i.e. the branching of the brachial nerve into n. brachialis inferior and n. brachialis superior. Phox2-RCAS-infected embryos were analysed at E8 for the expression of (B) TH, (C) DBH, (D) SCG10, (E) NF160, (F) VAChT and (G) ChAT in the brachial nerve by in situ hybridisation. The outline of the nerves is indicated by dashed lines. Magnification bars, 100 μm and 50 μm in B-E and F,G, respectively.

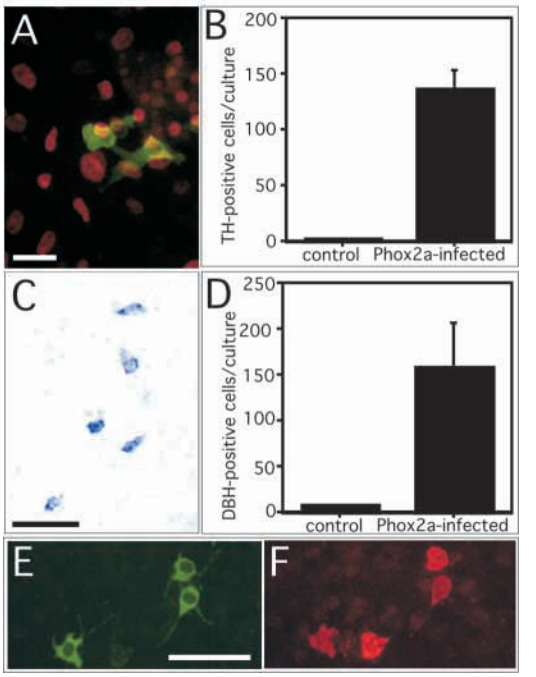

Fig. 4. Phox2a expression in cultures of E5 sensory DRG cells elicits the development of neurons expressing noradrenergic and pan-neuronal genes. (A) Double-immunostaining for Phox2a (red) and TH (green) in E5 DRG cultures after 5 days in culture. Phox2a-IR is restricted to the nuclei of infected cells, some of which co-express TH. (B) The number of TH-positive cells in uninfected control cultures and Phox2a-RCAS-infected cultures is quantified (mean ± s.e.m. of 13 platings in 3 independent experiments). (C) DBH-expressing cells in Phox2a-RCAS-infected cultures revealed by in situ hybridisation. (D) Quantification of the number of DBH-positive cells in uninfected control cultures and Phox2a-RCAS-infected cultures (mean ± s.e.m. of 9 platings in 2 independent experiments). (E,F) Coexpression of TH (E; green) and neuron-specific Hu-antigens (F; red) is shown by double immunostaining. Note that sensory neurons present in these cultures are Hu-positive and TH-negative. Magnification bars, 25 μm and 50 μm in A and C,E,F, respectively.

confirm this, cultures from dissociated E5 DRG were infected with Phox2a-RCAS and analysed for the presence of sympathoadrenergic cells. Whereas in control, uninfected cultures very few TH- or DBH-positive cells were present, in Phox2a-infected cultures a considerable number of cells expressing TH-IR or DBH mRNA developed (Fig. 4A,D). These cells had the morphology of immature sympathetic neurons rather than of sensory neurons and displayed in addition to noradrenergic also pan-neuronal properties, as revealed by double-immunostaining for TH and Hu-proteins (Marusich et al., 1994; Wakamatsu and Weston, 1997; Fig. 4E). Virtually all TH-positive cells coexpressed Hu proteins, although at lower levels as TH-negative sensory DRG neurons, also present in the cultures (Fig. 4). Phox2a expression did not increase the total cell number in DRG cultures (data not shown). Although the vast majority of cells in DRG cultures were infected and produced Phox2a, only few cells responded by noradrenergic differentiation (Fig. 4A-D).

It is unclear whether both Phox2 proteins or only Phox2a or Phox2b are (is) involved in sympathetic neuron development and control(s) the expression of terminal differentiation genes (Morin et al., 1997; Pattyn et al., 1999). Thus, it was of interest to investigate whether Phox2b would be able to elicit the same effects as Phox2a when overexpressed in the chick. Phox2b expression resulted in ectopic neurons in peripheral nerve, expressing the noradrenergic, pan-neuronal and cholinergic marker genes found in Phox2a-expressing embryos, *TH* (Fig. 5A), *SCG10* (Fig. 5B), *DBH*, *NF160*, *ChAT* and *VAChT* (not shown). Overexpression of Phox2b also induced Phox2a expression (Fig. 5C), as expected from the lack of Phox2a in *Phox2b*$^{-/-}$ mice (Pattyn et al., 1999). In view of the observed cross regulation between Phox2a and Phox2b in cranial sensory ganglia (Pattyn et al., 1997), we were interested to know whether the ectopic expression of Phox2a would result in the induction of endogenous Phox2b. The presence of Phox2b-expressing ectopic cells in brachial nerves of Phox2a-

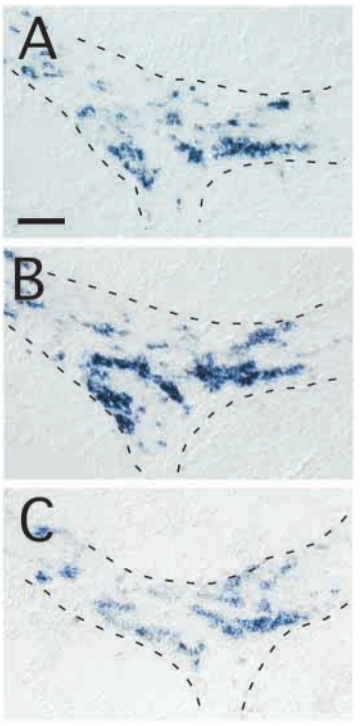

Fig. 5. Phox2b expression in the chick embryo results in ectopic cells expressing noradrenergic and pan-neuronal marker genes and *Phox2a*. Phox2b-RCAS-infected embryos were analysed at E8 for the expression of (A) TH, (B) SCG10 and (C) Phox2a in the brachial nerve by in situ hybridisation. The outline of the nerves are indicated by dashed lines. Magnification bar, 100 μm.

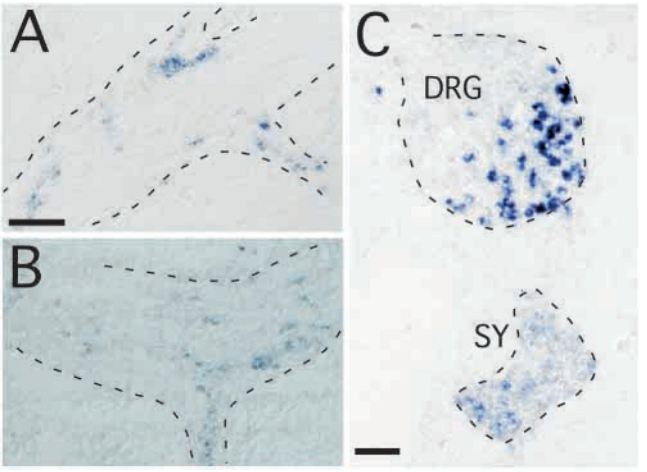

Fig. 6. Phox2a expression in the chick embryo induces the expression of Phox2b and c-ret in ectopic cells in the brachial nerve. Phox2-RCAS-infected embryos were analysed at E8 for the expression of (A) Phox2b and (B) c-ret in the brachial nerve by in situ hybridisation. The weak intensity of the c-ret in situ hybridisation signal (B) corresponds to the low level expression observed in sympathetic ganglia at this stage of development, as compared to the DRG (C). (C) In situ hybridisation for c-ret of a section from an uninfected E8 embryo. The outline of brachial nerve in A,B and of DRG and sympathetic ganglion (SY) in C is indicated by dashed lines. Magnification bars, 100 μm.

RCAS-infected embryos (Fig. 6) demonstrates that Phox2b can be positively regulated by Phox2a also in autonomic neurons and their precursors, although expression of the endogenous gene does not depend on Phox2a (Hirsch et al., 1998).

The elimination of *Phox2* genes in transgenic animals resulted in a lack of c-ret expression that seems causally related to the death of neurons observed in the superior cervical ganglion and the enteric nervous system (Pattyn et al., 1999). Thus, we investigated the effect of Phox2a on c-ret expression. Ectopic c-ret-expressing cells were found in the brachial nerve, albeit only in low numbers (Fig. 6), which may be explained by the low-level expression of c-ret in chick sympathetic ganglia, as compared to sensory DRG, at this stage of development (Fig. 6). Similar results were obtained upon overexpression of Phox2b. The c-ret expression in Phox2-induced ectopic neurons shows that *Phox2* genes are sufficient to elicit c-ret expression in some of the cells.

DISCUSSION

The Phox2 paired homeodomain proteins are essential for the generation of noradrenergic neurons in the peripheral and central nervous system. Overexpression of Phox2a and Phox2b in the peripheral nervous system now demonstrates that Phox2 proteins are sufficient to promote the development of sympathetic neurons and control, directly or indirectly, a broad spectrum of neuronal genes.

The analysis of the PNS from Phox2-deficient animals suggested two major functions of Phox2 proteins, the control of noradrenergic differentiation and cell survival (Morin et al., 1997; Pattyn et al., 1999). The survival effect of *Phox2* genes may be mediated through effects on the expression of the neurotrophic factor receptor c-ret in the vagal-enteric lineage, which gives rise to sympathetic neurons of the SCG and enteric neurons (Durbec et al., 1996). The Phox2-induced generation of noradrenergic cells in neural crest cultures and in the chick embryo supports and extends previous conclusions based on loss-of-function experiments. The results demonstrate that *Phox2* genes are not only essential but sufficient to elicit noradrenergic development, both in vitro and in vivo.

Interestingly, not only adrenergic but also the pan-neuronal genes *NF160* and *SCG10* and the cholinergic marker genes *ChAT* and *VAChT* are activated. The pattern of noradrenergic, pan-neuronal and cholinergic genes expressed by Phox2-induced cells is characteristic for paravertebral sympathetic neurons (Ernsberger et al., 1995, 1997) and differs from that of parasympathetic and enteric neurons. Parasympathetic ciliary ganglia contain TH-expressing cells but this cell population represents a very low proportion (0.5%) of the total ganglion cells (Teitelman et al., 1985; Iacovitti et al., 1985). TH-expressing adrenergic neurons or neural precursors are never observed in the avian enteric nervous system, either during development or after maturity (Smith et al., 1977; Le Douarin et al., 1978; Mackey et al., 1988). In agreement with the published data, we did not observe expression of TH or DBH mRNA in enteric SCG10-expressing neurons of the oesophagus, present in cross-sections of E8 embryos (not shown). Taken together, it seems appropriate to term the Phox2-induced ectopic cells sympathetic neurons, although they may differ in some aspects from bona fide sympathetic neurons.

Cholinergic marker genes are expressed in a subpopulation of paravertebral sympathetic ganglion neurons from E6 onwards (Ernsberger et al., 1997; M. S., unpublished observation). This early expression indicates that cholinergic

differentiation is not under the control of target-derived signals. ChAT and VAChT expression by a subpopulation of sympathetic neurons in paravertebral ganglia and ectopic neurons in peripheral nerve implies that extrinsic signals inducing cholinergic differentiation are available in both locations. Alternatively, cholinergic marker genes may be induced intrinsically, under the participation of Phox2 proteins, or by an autocrine mechanism.

Effects of Phox2a overexpression were only observed in a subpopulation of cells in the peripheral nerve, neural crest and DRG. Neural crest cultures contain fate-restricted precursors for neurons, glia and melanocytes, partially restricted e.g. neuron-glia precursor cells and unrestricted, multipotent precursor cells (Sieber-Blum and Cohen, 1980; Baroffio et al., 1988; Henion and Weston, 1997). In addition, there is evidence for single neural crest cells giving rise in vivo or in vitro to a restricted array of derivatives, as sensory neurons and glia (Frank and Sanes, 1991; Fraser and Bronner-Fraser, 1991), sensory neurons only (Sieber-Blum, 1989; Frank and Sanes, 1991) or sympathoadrenergic and non-neuronal/glial cells (Baroffio et al., 1988; Fraser and Bronner-Fraser, 1991; Duff et al., 1991). In neural crest cultures, multipotent cells are present in low numbers and only during the initial phase of the culture, as compared to fate-restricted cells (Henion and Weston, 1997). BMPs elicit the expression of *Phox2a* and sympathoadrenergic marker genes in a low proportion of neural crest cells and only when applied during the first day(s) in culture (Reissmann et al., 1996). Together with the present observations that Phox2a expression induces TH expression in a low proportion of infected cells, this suggests that mainly multipotent precursor cells are responsive to Phox2a and BMPs in neural crest cultures. In the absence of Phox2a or BMPs these cells are assumed to differentiate to other derivatives of multipotent cells, i.e. glia or melanocytes.

Likewise, there is evidence for the presence of multipotent neural crest precursor cells in peripheral ganglia and nerve. The potential of these cells to differentiate into sympathoadrenergic cells (Le Lievre et al., 1980; Xue et al., 1985; Rohrer et al., 1986; Duff et al., 1991) is revealed either after culturing the cells (Xue et al., 1985; Duff et al., 1991) or after back-transplantation of the ganglia into the neural crest migration pathway of younger embryos (Le Lievre et al., 1980; Schweizer et al., 1983). These cells are prevented from realizing their potential by the environment of peripheral ganglia, but are detectable as dormant precursors for a large period of embryonic development (Xue et al., 1985; Duff et al., 1991). Clonal analysis of cells derived from postmigratory trunk crest derivatives, including peripheral ganglia (Duff et al., 1991), gut (Sextier-Sainte-Claire Deville et al., 1994) or skin (Richardson and Sieber-Blum, 1993), revealed the presence of cells with restricted potential and of different multipotent cells, some of which give rise to clones containing sensory neurons and pigment cells in addition to adrenergic neurons. Recent findings demonstrated that ectopic expression of neurogenin bHLH transcription factors in the chick embryo induces the expression of sensory neuron-specific genes in neural crest cells of peripheral nerve (Perez et al., 1999). In view of the low proportion of Phox2-responsive cells in DRG and nerve, it is very likely that multipotential or partially restricted neural crest precursor cells in DRG and peripheral nerve represent the responsive cell population detected in Phox2-overexpression experiments, whereas fate-restricted glial or sensory neuron precursors would be refractory to the action of Phox2 proteins. Similarly, the increased size of sympathetic ganglia, observed in Phox2-infected embryos, is most likely due to an action on neural crest precursor cells that do not realize their potential to differentiate to sympathetic neurons during normal development. This notion is supported by recent observations that many TH-negative neural crest cells are present in sympathetic ganglia at least up to E4.5 (C. Schneider and H. R., unpublished observation). Also in the mammalian PNS, multipotent, self-renewing precursor cells with the potential to generate neurons and glia were recently demonstrated in peripheral nerve until late stages of fetal development (Morrison et al., 1999). The normal fate of these precursor cells is presently unclear. As the derivatives of multipotent stem cells in mammalian peripheral nerve include Schwann cells and myofibroblasts, it has been suggested that these stem cells would contribute to peripheral glia and myofibroblast derivatives in the nerve, including perineurium and epineurium (Morrison et al., 1999).

What is the cellular mechanism of action of Phox2 proteins? There are several major, nonexclusive possibilities, as (i) increased survival or proliferation of neural crest precursor cells with the potential to differentiate to sympathetic neurons, (ii) specification of cell fate of multipotent precursors to the sympathoadrenal lineage or (iii) induction of the expression of terminal differentiation genes in prespecified sympathoadrenal precursor cells. It is very difficult to distinguish between these possibilities in the present experimental model. However, increased survival or proliferation of sympathoadrenergic precursor cells is an unlikely explanation for the generation of ectopic sympathetic neurons in DRG and nerve: precursor cells with the potential to differentiate to sympathetic neurons are present until at least E15 in the chick DRG (Xue et al., 1985). As these precursors are not able to differentiate during normal development in the environment of the DRG, increased survival or proliferation would not lead to the generation of sympathetic neurons. An action of *Phox2* genes on prespecified sympathoadrenergic precursor cells in neural crest and postmigratory neural crest derivatives cannot be excluded, in view of the evidence for early fate restriction in neural crest cells (Henion et al., 1997; Raible and Eisen, 1994). However, as direct evidence for the presence of prespecified sympathoadrenergic precursor cells in neural crest, DRG and peripheral nerve is lacking, whereas multipotent cells could be demonstrated (Duff et al., 1991; Morrison et al., 1999) it seems more likely to us that *Phox2* genes act mainly on multipotent trunk neural crest precursor cells, specifying cell fate towards the sympathoadrenergic lineage by the induction of pan-neuronal and noradrenergic differentiation genes.

The in vivo findings leave the question open whether Phox2 proteins require a previous action of BMPs to induce the expression of pan-neuronal genes and *DBH*, as suggested for sympathetic ganglia (Lo et al., 1999). BMPs are expressed in the developing vertebra and in limb mesenchyme during later development (Lyons et al., 1995; U. E., unpublished observations), but it is unclear whether this occurs before cells are infected by Phox2-RCAS and whether mesenchyme-derived BMPs reach the precursor cells in the DRG or peripheral nerve. BMPs are absent in Phox2a-infected cultures of neural crest and DRG, suggesting that *Phox2* genes are

sufficient to induce pan-neuronal and noradrenergic genes and do not require a previous action of BMPs. These results are in agreement with the notion that *Phox2* genes are involved in the signal transduction pathway of BMPs in this neuronal lineage.

How is the effect of Phox2a mediated? In the case of DBH and TH, the effects may be explained by a direct activation of these terminal differentation genes, as both DBH and TH promoters contain binding sites for Phox2a and Phox2b and Phox2a/b can transactivate the DBH and/or TH promotor (Zellmer et al., 1995; Swanson et al., 1997; Lo et al., 1999; Yang et al., 1998). The present analysis reveals that not only noradrenergic but also pan-neuronal and cholinergic genes are expressed in ectopic cells. Pan-neuronal genes and noradrenergic genes are expressed within a short developmental time period after the onset of *Phox2* gene expression in sympathetic ganglia (Ernsberger et al., 1995; U. E., unpublished observation), suggesting a common control mechanism. Consistent with this, Phox2a was discovered by its binding to the promotor of the pan-neuronal *NCAM* gene (Valarché et al., 1993). However, noradrenergic and pan-neuronal gene expression can experimentally be uncoupled (Christie et al., 1987; Groves et al., 1995; Sommer et al., 1995; Lo et al., 1998), arguing for independent subprograms. In line with these findings, recent in vitro studies demonstrated that forced expression of *Phox2a* induces the expression of *TH* but not *DBH* and pan-neuronal genes in mammalian neural crest cultures (Lo et al., 1998, 1999). *Phox2* genes were shown, however, to be essential for the expression of noradrenergic and neuronal genes in response to BMPs and forskolin (Lo et al., 1999). The Phox2-induced expression of pan-neuronal and noradrenergic marker genes that we observe in the chick suggests that co-factors required for the induction of noradrenergic and pan-neuronal genes are present in trunk neural crest precursor cells in vivo, but may not be available in vitro, depending on the culture conditions. In view of the large time difference between the onset of Phox2 expression in primary sympathetic ganglia and the onset of cholinergic differentiation it may seem unlikely that *Phox2* genes are involved in the control of the cholinergic locus. On the contrary, Phox2a is continuously expressed throughout embryonic sympathetic neuron development and later functions of Phox2a may include a participation in the regulation of cholinergic differentiation.

It thus seems that several subprograms involved in the regulation of neuron differentiation, neurotransmitter phenotype and neuron survival are controlled directly or indirectly by Phox2 proteins in sympathetic neuron precursor cells. The molecular details of the pathways involved remain to be characterized. Although forced expression of either Phox2a or Phox2b are sufficient to elicit the expression of noradrenergic and pan-neuronal genes, the cross-regulations between Phox2 proteins shown by the present and previous studies (Pattyn et al., 1997, 1999) leave the question open whether either one or both are required in our system. In the knockout experiments (Morin et al., 1997; Pattyn et al., 1999) Phox2a, but not Phox2b, was found to be dispensable for noradrenergic differentiation in the sympathoadrenal lineage. Hence, Phox2b is able to promote sympathoadrenal differentiation in the absence of Phox2a, but whether this is also true for Phox2a has not been clarified.

As in most neuronal lineages, in particular the autonomic nervous system, the elimination of *Phox2* genes results in the early death of the cells, only few candidate downstream genes, i.e. *c-ret* and *DBH*, were identified. The present experiments support and extend the conclusions drawn from the loss-of-function experiments, suggesting that Phox2 proteins control a much larger aspect of autonomic neuron development than anticipated, including pan-neuronal and cholinergic traits. This notion may be verified by conditional loss-of-function experiments at later stages of sympathetic neuron development or in the adult.

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