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Lhx9 and *Lhx9α:* Differential Biochemical Properties and Effects on Neuronal Differentiation

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ABSTRACT

The Lhx9 LIM-homeodomain transcription factor and its truncated isoform Lhx9α are generated by alternative splicing of the *Lhx9* gene. Here we investigated the differential functional properties of these two isoforms. Lhx9α, which lacks parts of the homeodomain, was unable to bind DNA in EMSA experiments, but was able to associate with CLIM cofactors in GST pull-down assays. In transfection experiments in PC12 cells, Lhx9α fusion constructs systematically showed a nuclear localization, as opposed to Lhx9 fusion constructs, which also localized to the cytoplasm. Moreover, Lhx9 increased NGF-induced neuronal differentiation of PC12 cells. Lhx9α, on the other hand, did not significantly increase neuronal differentiation but had an effect on the morphology of PC12 cells. Finally, as tested by RT-PCR experiments on transfected PC12 cells, Lhx9 was not able to induce the transcription of Lhx9α. Our results show significantly different functional properties for Lhx9 and Lhx9α, and suggest that Lhx9α can compete away limiting amounts of nuclear CLIM cofactors. Thus, Lhx9 and Lhx9α isoforms could be implicated in regulating various aspects of neuronal differentiation.

INTRODUCTION

LIM DOMAIN-CONTAINING PROTEINS represent a large family including LIM-homeodomain (LIM-HD) developmental transcription factors, LIM-only (LMO) nuclear or cytoplasmic factors, and LIM-kinases (reviewed in Bach, 2000; Rétaux and Bachy, 2002). The functions of these various LIM proteins are diverse, from patterning and cell specification during development to cytoskeletal regulation and control of cell division. Among them, the LIM–HD transcription factors and the LMO nuclear factors exert their function via an interaction of their LIM domains with cofactors named NLI/Ldb/CLIM (Agulnick *et al.,* 1996; Jurata *et al.,* 1996; Bach *et al.,* 1997) or with other factors (e.g., Bach *et al.,* 1999). Therefore, the LIM domains are generally regarded as protein–protein interaction motifs, which constitute scaffolds for the formation of higher order regulatory complexes.

There are 13 LIM–HD family members in mammals, which are distributed into six subgroups, and which all have orthologs in nonmammalian vertebrates and invertebrates (Failli *et al.,* 2000; Bachy *et al.,* 2002). They are expressed in highly specific patterns throughout embryonic development, particularly in the nervous system where they are involved in regional and neuronal cell-type specification. Among other examples, *Isl1* controls motorneuron specification in the spinal cord (Pfaff *et al.,* 1996), *Lmx1b* controls the serotoninergic phenotype (Cheng *et al.,* 2003; Ding *et al.,* 2003), and *Lhx7* induces forebrain cholinergic neuron differentiation (Zhao *et al.,* 2003). To exert their transcriptional control on such various aspects of neuronal differentiation, LIM–HD factors interact with Ldb1/Ldb2 (or CLIM1/CLIM2), which are also expressed throughout the developing brain (Bach *et al.,* 1997). Due to their capacity of self-dimerization (Jurata and Gill, 1997), these cofactors can bridge two LIM–HD proteins together, and allow the formation of transcriptionally active heterotetrameric (or even hexameric) complexes composed of 2LIM-HD:2CLIM proteins. Studies in *Drosophila* have shown that any disruption of the LIM-HD:CLIM complex by factors such as LMO proteins which are able to compete with LIM–HD for CLIM interaction, also disrupts the normal function of the LIM–HD factor in a developmental event (Milan *et al.,* 1998; Milan and Cohen, 2000). Because LMO, LIM–HD and cofactors are often coexpressed in

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the same cells, a tightly regulated equilibrium between the expression levels and activity levels of the different partners is present for the developmental process to take place correctly (Weihe *et al.,* 2001; Becker *et al.,* 2002; Ostendorff *et al.,* 2002; Hiratani *et al.,* 2003).

The mouse *Lhx9* gene is able to generate two alternative transcripts encoding two isoforms, Lhx9 and Lhx9α (Failli *et al.,* 2000). Whereas Lhx9 presents the classical features of a LIM–HD factor, Lhx9α is truncated at the level of the third helix of its homeodomain. Moreover, the developmental expression patterns of the two transcripts show significant differences in levels and distribution. Therefore, we previously suggested that Lhx9α could function as an endogenous dominant-negative form of Lhx9 during development, and could regulate and/or refine in space and time the transcriptional effects of Lhx9 (Failli *et al.,* 2000), in a manner similar to the competition between LIM–HD and LMO proposed in Drosophila (Milan *et al.,* 1998).

Here, we further characterize the biochemical properties of Lhx9α, and show that this truncated isoform behaves like a nuclear LMO protein. In addition, we assess the respective effects of Lhx9 and Lhx9α on neuronal differentiation in cell culture experiments, and show that they have distinct effects on the differentiation of PC12 cells.

MATERIALS AND METHODS

In vitro *protein–protein interaction assays*

PCR products of full-length Lhx9 and Lhx9α were ligated in frame into the EcoRI/NcoI sites of the pGEX-KG bacterial expression vector to yield glutathione S-transferase (GST) fusion proteins. The *in vitro* protein–protein interaction assays with ^{35}S-methionine labeled, *in vitro* transcribed-translated CLIM1 and CLIM2 proteins were performed as described previously (Bach *et al.,* 1997).

Electrophoretic mobility shift experiments (EMSA)

EMSA experiments and EMSA supershift assays were performed as described previously (Bach *et al.,* 1997) using ^{32}P-labeled oligonucleotides that encompass the Lhx3 binding site on the αGSU promoter (Roberson *et al.,* 1994; Bach *et al.,* 1995) and bacterially expressed GST-fusion proteins.

Culture of PC12 cells

PC12 cells were maintained in RPMI–glutamax culture medium containing 10% heat-inactivated horse serum and 5% heat-inactivated foetal veal serum. For NGF treatment (2.5S, Promega, Madison, WI, final concentration 50 ng/ml), the serum content was reduced to 1% total.

For transfection experiments, 2.10^6 cells were suspended in 400 μl Opti-MEM and electroporated in the presence of 10 μg DNA on a Biorad electroporator. Cells were then plated onto 24-well plates at a density of $1–5.10^5$ cells/well on glass coverslips coated with Matrigel or with poly-L lysine/poly-ornithine/laminine. After 3 days of NGF treatment cells were fixed with 4% paraformaldehyde and processed for immunofluorescence and imaging.

For RT-PCR experiments, total RNA was extracted from $\sim 10.10^6$ cells using Trizol (Invitrogen, Carlsbad, CA). Random-primed, reverse-transcribed cDNA (AMV reverse transcriptase, Roche, Indianapolis, IN) was used as template for PCR using specific oligonucleotides primers (18–20 mers, sequence available on request) designed on the sequences of mouse (or rat when available) LIM-HD and CLIM cDNAs.

Lhx9 and Lhx9α expression constructs

The full-length coding sequences of Lhx9 and Lhx9α were amplified by PCR and subcloned in frame as GFP or myc-tag fusions into the pEGFP-N3 (Clontech, Palo Alto, CA) or the pCS2-MT (a gift of David Turner) expression vectors.

Imunofluorescence staining on PC12 cells

Fixed cells were rinsed with PBS and primary antibody incubation was performed during 2 h at room temperature in PBS containing 0.1% Triton and 0.5% BSA (PBT). A monoclonal mouse anti-GFP (Roche) was used at 1/500 and a monoclonal anti-myc (9E10) FITC-conjugate (Sigma, St. Louis, MO) was used at 1/200. Secondary antibodies were added in PBT (goat antimouse-Alexa488 or -Alexa594, Molecular Probes, 1/200) for 1 h and washed again. If needed, cells were counterstained with Alexa-594 phalloidin and DAPI (Molecular Probes). Coverslips were mounted with PPD-glycerol antifading medium and observed on a Nikon E800 fluorescence microscope equipped with a DXM1200 camera. Images were occasionally corrected for brightness/contrast and mounted using Adobe Photoshop (Adobe Systems, San Jose, CA).

Differentiation assay

For the neuronal differentiation assay of NGF-induced PC12 cells, a minimum of nine coverslips containing at least 150 transfected cells and obtained from three independent experiments were counted. Cells were considered to exhibit signs of neuronal differentiation if at least one of their neurite was equal in length or longer than the soma size. Statistical comparison was carried out using a Student's *t*-test.

RESULTS

Lhx9a is able to interact with cofactors but does not bind DNA

Lhx9 and Lhx9α are identical in their N-terminal amino acid sequence including LIM domains, linker, and the two first helices of the homeodomain. The rest of their sequences is different, in that Lhx9α is truncated at the level of the third helix of the homeodomain, and shows a distinct C-terminus sequence and 3′UTR sequence (Failli *et al.,* 2000, schematized in Fig. 1A). Because Lhx9α lacks the third helix of the homeodomain which has previously been identified as crucial for homeodomain–DNA interactions (e.g., Kissinger *et al.,* 1990), we first tested and compared the DNA binding capacities of the two Lhx9 isoforms using EMSA experiments, with a probe containing the LIM–HD binding site of the αGSU promoter. This sequence is recognized at least by Lhx2, the Lhx9 paralog, and by Lhx3 (Roberson *et al.,* 1994; Bach *et al.,* 1995). Indeed, similar to Lhx3, Lhx9 was not only able to interact with this sequence (Fig. 1B) but also to form slower migrating complexes

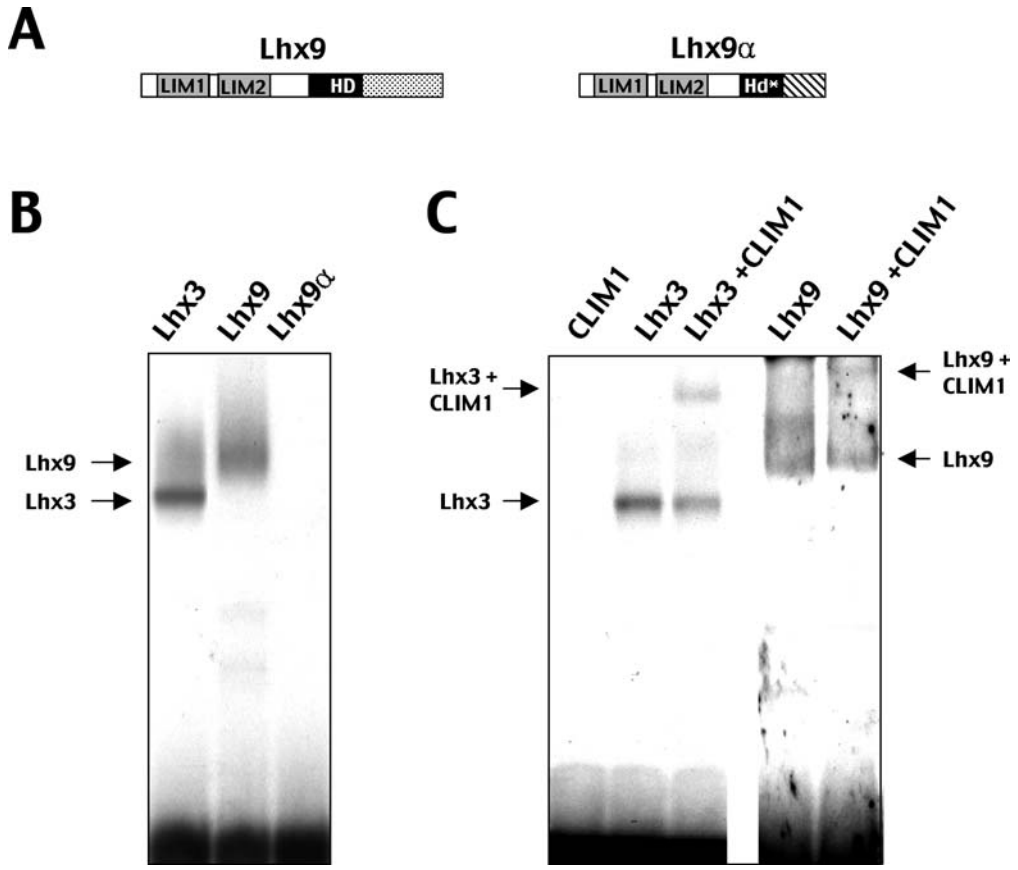

FIG. 1. DNA-binding capacities of Lhx9 and Lhx9α. (**A**) A schematic representation of the structure of the two Lhx9 isoforms. Both isoforms are identical in their LIM domains (LIM1 and LIM2, gray boxes) and the beginning of their homeodomains (HD, black box), as indicated by the gray shading between dotted lines. Note that the homeodomain of Lhx9α is truncated (asterisk) and that they differ in their C-terminal sequence (indicated by different fill patterns). (**B**) An EMSA experiment showing the interaction of Lhx3 and Lhx9 but not Lhx9α on the oligonucleotide probe. (**C**) Shows the formation of a ^{32}P-labeled complex supershift in the presence of CLIM1 for both Lhx3 and Lhx9.

with the CLIM1 protein while bound to DNA in supershift experiments (Fig. 1C), indicating that Lhx9 can form ternary complexes on DNA with CLIM cofactors. Conversely, Lhx9α was not able to form a complex with the oligonucleotide probe (Fig. 1B, right lane), showing that the absence of the third helix of the homeodomain abolishes its capacity to bind DNA.

Because Lhx9 and Lhx9α both contain two identical LIM domains we tested their capacity to bind CLIM cofactors in GST pull-down experiments. The results demonstrate that both Lhx9 and Lhx9α interact with CLIM1 and CLIM2 proteins with comparable affinity (Fig. 2A–B). Thus, similar to LMO proteins, Lhx9α associate with CLIM cofactors but does not bind to DNA.

Lhx9, Lhx9a, and neuronal differentiation

We have previously reported that *Lhx9* and *Lhx9a* are mostly coexpressed in the same brain regions, *Lhx9a* being expressed at lower levels and significantly later in development. Given that LIM–HD factors are commonly involved in neuronal specification, we next sought to investigate whether the two Lhx9 isoforms had differential effects on the neuronal differentiation process. To this end, we used PC12 cells (a rat pheochromocytoma cell line), taking advantage of this cell line property to differentiate into neurons after NGF treatment (Fig. 3A–B). To validate the PC12 cell culture system as a tool to assess the effect of a LIM–HD factor, we first tested whether these cells express the

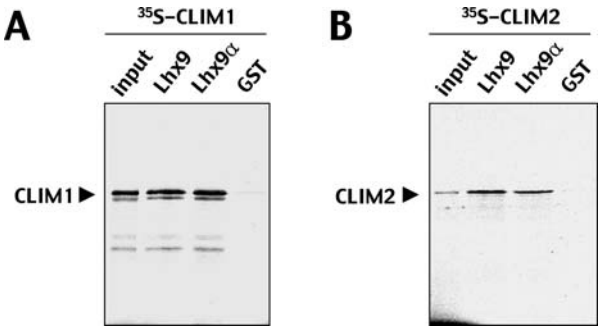

FIG. 2. CLIM-binding capacities of Lhx9 and Lhx9α. (**A**, **B**) Present autoradiograms of GST pull-down experiments where ^{35}S-labeled CLIM1 (**A**) or CLIM2 (**B**) strongly interact with bacterially expressed GST-fusion of Lhx9 and Lhx9α. The input lane shows 10% of the total ^{35}S-labeled protein input, and the control GST lane shows that GST itself does not interact with the CLIMs.

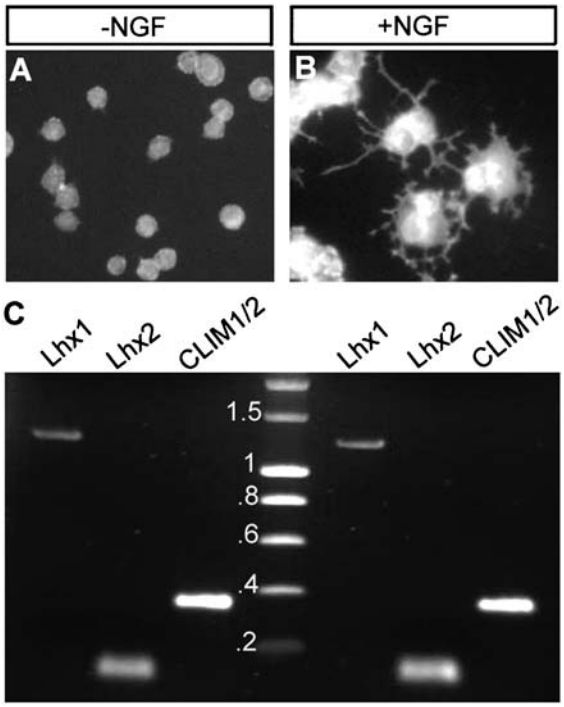

FIG. 3. PC12 cells express proteins of the LIM-HD network. (**A**, **B**) Show high magnification fluorescence photomicrographs of PC12 cells not treated (**A**) or treated (**B**) with NGF during 72 h. Cells were counterstained with Alexa-594 phalloidin to visualize the actin cytoskeleton and the neurites, together with a DAPI nuclear stain. (**C**) Shows the result of RT-PCR experiments performed on total RNA extracted from control or NGF-treated PC12 cells. The identity of PCR products are indicated and run at the expected size according to the pairs of primers used (1.2 kb for *Lhx1*, 160 bp for *Lhx2*, and 350 bp for *CLIM*).

components of the LIM–HD protein network, that is, CLIM cofactors and at least some LIM–HD factors. RT-PCR analysis on PC12 cells with or without NGF treatment showed that under both conditions these cells express mRNAs encoding CLIM cofactors (the oligonucleotide primers used for PCR amplify both CLIM1 and CLIM2) and the LIM–HD factors Lhx1 and Lhx2 (Fig. 3C). Other LIM–HD members tested included Lhx5, Lhx9, Lhx9α, and Lmx1a, which were not detected in these RT-PCR experiments (data not shown and Fig. 6B).

In a first series of experiments, and to further characterize the functional properties of the two isoforms, Lhx9–GFP and Lhx9α–GFP fusion constructs were electroporated into PC12 cells. We found a clearly distinct subcellular localization of the two fusion proteins after GFP immunofluorescence staining: Lhx9-GFP was found abundantly throughout the cell nucleus, cytoplasm, and neurites, whereas Lhx9α–GFP was exclusively restricted to a punctate-like nuclear staining, both in the presence or absence of NGF (Fig. 4A and B). To discard the possibility of a distinct localization of Lhx9α due to the GFP tag, we transfected a Lhx9α–myc-tag construct. We found a similar, although slightly more diffuse, nuclear punctate-like staining after myc immunofluorescence labeling of the Lhx9α–myc fusion protein (Fig. 4C). The same results were observed when mouse primary cortical neurons were transfected with Lhx9–GFP or Lhx9α–myc-tag, respectively (data not shown). Thus, the strict nuclear localization of Lhx9α appeared like a specific feature of the truncated isoform. This conclusion was further supported by the fact that another LIM–HD protein, Lhx7, was also found to be distributed throughout the nucleus and cytoplasm of PC12 cells when fused to a myc epitope-tag (S. Rétaux and I. Bachy, unpublished observations).

In a next series of experiments, the effect of Lhx9 and Lhx9α on NGF-induced neuronal differentiation of PC12 cells was tested (Fig. 5). After 72 h of NGF treatment, PC12 cells transfected with a Lhx9–GFP fusion construct showed a significant 40% increase in neuronal differentiation compared to their GFP-transfected controls (Fig. 5A and B). Because Lhx9α–GFP fusion localized to the nucleus and therefore did not allow the visualisation of neuritic extensions, the effect of Lhx9α on neuronal differentiation was tested upon cotransfection of a Lhx9–myc-tag and a GFP construct, and was found intermediate and not significant compared to GFP and Lhx9–GFP in terms of neuronal differentiation (Fig. 5). However, it came out upon microscopic analysis that Lhx9- and Lhx9α-transfected cells presented significantly different morphological features. Lhx9- (as well as GFP-) transfected cells had "classical" neuronal characteristics, showing one or several neuritic extensions and a round or ovoid cell body, whereas a significant proportion of Lhx9α-transfected cells showed a flattened aspect (Fig.

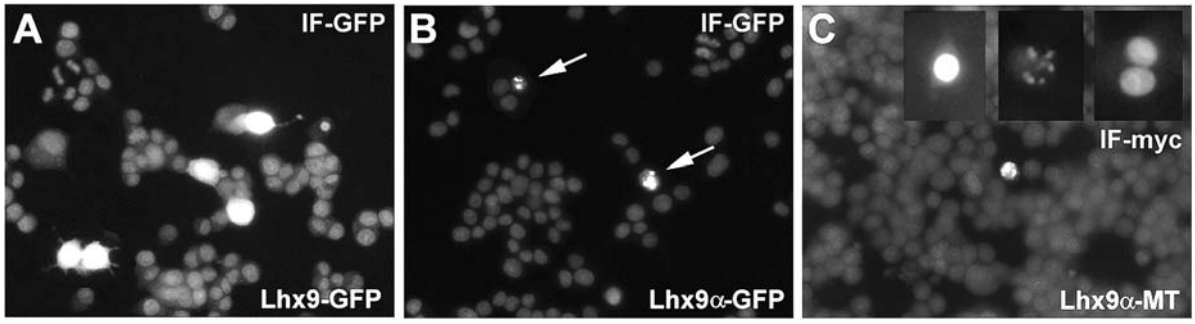

FIG. 4. Subcellular localization of Lhx9 and Lhx9α. (**A–C**) Immunofluorescence photomicrographs of PC12 cells transfected with a Lhx9-GFP (**A**), Lhx9α-GFP (**B**), or Lhx9α-myctag (**C**, and insets in **C**) fusion constructs, in the absence of NGF treatment, and counterstained with DAPI nuclear staining. Immunofluorescence is directed against GFP (**A,B**) or the myc epitope-tag (**C**) and reveals a clear nuclear localization for Lhx9α.

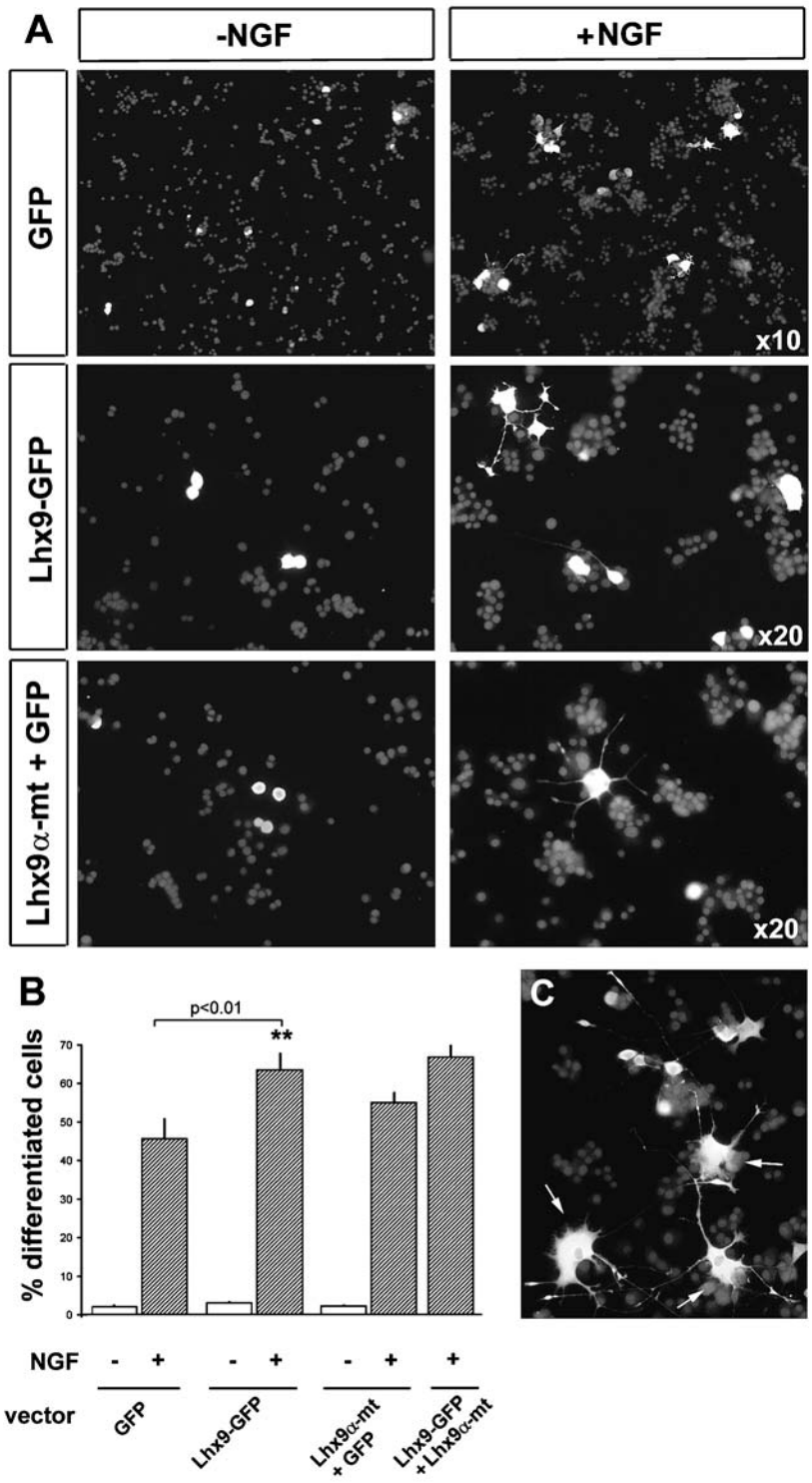

FIG. 5. Effect of Lhx9 and Lhx9α on NGF-induced differentiation of PC12 cells. (**A**) Immunofluorescence photomicrographs of PC12 cells transfected with the indicated constructs and cultured in the presence or absence of NGF. GFP immunofluorescence (IF-GFP) was used to outline the morphology of transfected cells. Cell nuclei were counterstained with DAPI. The magnification power of photomicrographs are indicated. (**B**) Histogram showing the quantification of the effect. (**C**) High-power photomicrograph showing a representative field where several *Lhx9α*-transfected cells with peculiar "flattened" morphology are present (indicated by arrows).

5C). Quantification showed that 24% of Lhx9α-transfected cells versus 10% of Lhx9-transfected cells presented this flattened morphological feature ($P < 0.01$, Student *t*-test). Thus, Lhx9 and Lhx9α had differential effects on the process of NGF-induced neuronal differentiation of PC12 cells.

Finally, to test a possible dominant-interfering action of Lhx9α on Lhx9, we cotransfected the two isoforms into PC12 cells and assessed NGF-induced neuronal differentiation. Upon cotransfection, we observed a 46% increase in neuronal differentiation which was not significantly different from the 40% increase observed with Lhx9 alone (Fig. 5B). In addition, 24% of cotransfected cells presented the "flat" phenotype after cotransfection.

Does Lhx9 regulate the transcription of Lhx9a?

During *Drosophila* wing disk development, the LIM–HD selector gene *apterous*, which is the *Lhx9* ortholog, induces the LMO inhibitor (dLMO) to terminate its own effect, and this regulation is crucial for the proper development of the wing (Milan and Cohen, 2000). In a last series of experiments, we tested whether a similar mechanism could be at work in our system of neuronal differentiation, that is, whether Lhx9 could positively regulate the transcription of Lhx9α. PC12 cells, which do not normally express either of *Lhx9* or *Lhx9α* transcripts (Fig. 6B), were transfected with Lhx9 or Lhx9α expression vectors and cultured in the presence of NGF before RT-PCR analysis. PCR primers were chosen either in the coding sequence or in the 3′UTR sequence of *Lhx9/9α* mRNAs, to discriminate between expression due to the transfected expression vector or to an endogenous transcriptional regulation (Fig. 6A). The results show that in the PC12 cell culture system, Lhx9 was not able to induce its own transcription or that of its isoform. However, and quite unexpectedly, in Lhx9α-transfected cells, a PCR product corresponding to the *Lhx9* transcripts was detected (Fig. 6B).

DISCUSSION

In this paper we have begun to investigate the possible functional and physiological relevance of the existence of two alternatively spliced isoforms for the Lhx9 transcription factor.

Lhx9α behaves like a nuclear LMO protein

With its two LIM domains and its truncated homeodomain, we had previously suggested that Lhx9α could be an endogenous dominant-negative or competitor isoform of the Lhx9 transcription factor. We now demonstrate that Lhx9α is able to bind the CLIM cofactors but not DNA, and although the subcellular localization information given by transfected epitope-tagged constructs is not fully dependable, the short isoform seems to localize preferentially to the nuclear compartment. Thus, Lhx9α has the capacity and the location to compete away limiting amounts of CLIM cofactors, similarly to nuclear LMO proteins

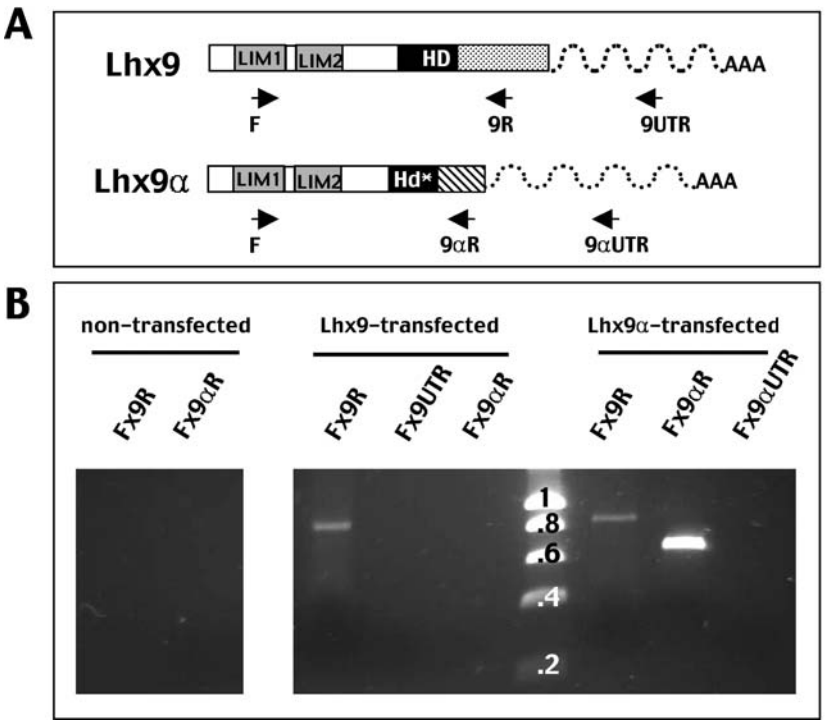

FIG. 6. Autoregulatory effect of Lhx9 and Lhx9a on their own transcription. (**A**) Schematic representation of the two transcripts, showing the localization of PCR primers chosen to discriminate between expression due to the transfected vector or to endogenous expression (drawing not to scale). (**B**) RT-PCR analysis of transcripts present in NGF-treated PC12 cells nontransfected or transfected with the indicated construct. A PCR product is present at the expected size in control lanes and in the experimental situation where Lhx9α-transfected cells induce the expression of *Lhx9* transcripts.

as previously reported (Milan *et al.,* 1998). More specifically, the punctate nuclear staining indicates that Lhx9α executes its regulation in specific nuclear subcompartments. Thus, the role of Lhx9α for functions of PML bodies and/or for nucleolar functions (reviewed in Eskiw and Bazett-Jones, 2002; Olson *et al.,* 2002) now needs further investigation.

In homeodomain-containing transcription factors, the third helix of the homeodomain has been classically recognized with a in DNA-binding activity. The result of our gel-shift experiments are fully compatible with this idea. However, and interestingly, the homeodomain of the antennapedia and engrailed homeoproteins have also been implicated in the possible nuclear export and secretion process of homeoproteins (Joliot *et al.,* 1991, 1997, 1998). As deduced from mutation and deletion analysis, the third helix of the homeodomain seems to be responsible and necessary for this export mechanism. Our experiments bring striking support to this hypothesis, with the observation that Lhx9 distributes to the entire cell (nucleus and cytoplasm), whereas Lhx9α, which precisely misses the third helix of the homeodomain, is restricted to the nucleus. Although we have not observed cell-to-cell transfer of the Lhx9 protein as reported for antennapedia or engrailed (e.g., Joliot *et al.,* 1998), we provide to our knowledge the first evidence for differential nuclear localization properties of two endogenously and naturally occurring isoforms of a homeodomain factor.

Lhx9/Lhx9α and neuronal differentiation

Nuclear LIM domain-containing factors (LIM–HD and nuclear LMO) are involved in cell specification and differentiation (Bach, 2000). On the other hand, some LMO factors are also powerful oncogenes (Rabbitts, 1998). It has therefore been hypothesized that in some human cancers caused by chromosomal LMO translocations the deregulation of LIM–HD activity by dominant-interfering LMO overexpression alters the proper maintenance of the differentiated state, and leads to a failure in control of cell proliferation. Indeed, in various systems, nuclear proteins consisting mainly of LIM domain proteins are implicated in the positive or negative control of cell differentiation: the mouse FHL2 factor promotes the differentiation of myoblasts (Martin *et al.,* 2002) and *Xenopus* LMO3 increases neurogenesis (Bao *et al.,* 2000), whereas LMO2 and LMO4 negatively regulate erythroid and mammary cell differentiation, respectively (Visvader *et al.,* 1997, 2001). The differential effects of Lhx9 and Lhx9α observed in this report regarding neuronal differentiation of PC12 cells are therefore consistent with a general role of the LIM–HD/LMO protein network in the control of cell differentiation, and also with their differential biochemical properties and subcellular localization: Lhx9 promotes neuronal differentiation, which fits well with the recent proposal that LIM–HD factors, together with proneural bHLH genes, could participate in the synchronization of cell cycle exit and cell specification (Lee and Pfaff, 2003), whereas Lhx9α rather has an effect on an aspect of cellular morphogenesis. In addition, in our experimental culture system, Lhx9α does not show any dominant-interfering effect on Lhx9-induced increase in neuronal differentiation, and Lhx9 does not interfere with Lhx9α-induced change in cell morphology, therefore suggesting an absence of competition between the two isoforms, and reinforcing the idea that they function in distinct pathways. In this sense, one could have hypothesized that Lhx9α, which is expressed *in vivo* at later embryonic stages than Lhx9, could be responsible for the refinement of the neuronal phenotype specified by the LIM–HD Lhx9. This is one of the reasons that prompted us to investigate whether Lhx9 was itself responsible for turning on the transcription of its alternative transcript. In our experimental conditions however, we did not detect any direct positive transcriptional activation of Lhx9α by Lhx9. However, although the molecular mechanisms remain unknown, our results suggest that Lhx9α may play a role in Lhx9 expression. Since mRNA encoding *Lhx9α* is transcribed at later stages of mouse embryonic development when compared directly with *Lhx9* (Failli *et al.,* 2000), the effects of Lhx9α on *Lhx9* mRNA expression appear to be most likely at the maintenance level.

CONCLUSION

The LIM–HD protein network is a complex system comprising multiple partners and involved in the regulation of many developmental events. This complexity is reinforced by the possibility to generate isoforms by alternative splicing mechanisms. Such isoforms have been described for other LIM–HD members: Lhx7a, truncated in the homeodomain similarly to Lhx9α, has not been functionally studied (Grigoriou *et al.,* 1998); Isl1α and Isl1β differ by their carboxy-terminal sequence, have differential transactivation activities and undergo differential phosphorylation (Ando *et al.,* 2003). There are also N-terminal alternatively spliced Lhx3 isoforms, which differ in terms of gene activation properties (Sloop *et al.,* 1999). The example of Lhx9/Lhx9α reported here provide insights into the functional/physiological significance of the existence of these multiple isoforms.

ACKNOWLEDGMENTS

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