A family of LIM domain-associated cofactors confer transcriptional synergism between LIM and Otx homeodomain proteins

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The essential roles of LIM homeodomain proteins in cell fate determination during development have been demonstrated in organisms as divergent as *Drosophila* and higher mammals. We have isolated murine cDNAs encoding two highly homologous proteins that specifically interact with the LIM domains of P-Lim/Lhx3 and several other LIM homeodomain factors. Transcripts encoding these factors can be detected as early as mouse E8.5, with maximal expression observed in regions of the embryo in which the LIM homeodomain factors P-Lim/Lhx3, Isl-1, and LH-2 are selectively expressed. These proteins can potentiate transactivation by P-Lim/Lhx-3 and are required for a synergistic activation of the glycoprotein hormone α -subunit promoter by P-Lim/Lhx3 and a pituitary Otx class homeodomain transcription factor, with which they also specifically associate. Our results link LIM homeodomain proteins and members of the Otx class of transcription factors in gene activation events during embryogenesis via the actions of specific cofactors.

[**Key Words:** LIM domain; coactivator; LIM and Otx homeodomain factors; protein-protein interaction; synergistic transcriptional activation]

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The LIM domain is a conserved cysteine- and histidine rich structure of two tandemly repeated zinc fingers, first identified in homeodomain transcription factors (for review, see Sánchez-García and Rabbitts 1994; Dawid et al. 1995). LIM domains were subsequently found in proteins that do not contain homeodomains, for example, in LIM-only proteins that contain essentially only one or several LIM domains, or in the LIM kinases LMK-1 and LMK-2 in which two LIM domains are associated with a protein kinase domain (Bernard et al. 1994; Mizuno et al. 1994). The LIM domain has been shown to be a protein-protein interaction domain (Feuerstein et al. 1994; Schmeichel and Beckerle 1994) and in LIM homeodomain factors, it has been implicated in the inhibition of DNA binding (Sánchez-García et al. 1993; Taira et al. 1994), but it has also been shown to be required for synergistic activation of genes by LIM homeodomain proteins acting with other transcription factors (German et al. 1992; Bach et al. 1995).

Members of the LIM homeodomain factor family exhibit distinct patterns of developmental expression. Isl-1 is expressed at high levels in hindbrain, forebrain, ven-

tral neural tube, dorsal root ganglia (DRG), and trigeminal ganglia. In the ventral neural tube, Isl-1 is the first marker of developing motor neurons (Ericson et al. 1992; Yamada et al. 1993) and a mutation of the *Isl-1* gene leads to lack of motor neurons and interneurons (Pfaff et al. 1996). The LIM homeoprotein LH-2 is detected in discrete regions of the developing nervous system and in a subset of lymphoid tissues (Xu et al. 1993), whereas P-Lim/Lhx3/m-Lim3 is expressed highly and specifically early in development of the pituitary gland and the ventral neural tube (Seidah et al. 1994; Bach et al. 1995; Zhadanov et al. 1995). LH-2 and P-Lim/Lhx3 have been shown to be capable of binding to and activating the promoter of the glycoprotein hormone α -subunit gene (α GSU), a marker of early pituitary development, in transient transfection assays (Roberson et al. 1994; Bach et al. 1995). In the developing pituitary gland, P-Lim/Lhx3 mRNA is first detected at mouse embryonic (E) day 8.5–9.0 in the invagination of the somatic ectoderm in the roof of the oral epithelium, known as Rathke's pouch. The importance of P-Lim/Lhx3 in pituitary development was established by demonstrating that four of the five hormone-producing cell lineages in the anterior pituitary gland and expression of several markers including α GSU are lost in *Lhx3/P-Lim* (–/–) mice (Sheng et al. 1996). Several other homeodomain factors are highly expressed

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in early Rathke's pouch, including P-OTX/Ptx1, a homeoprotein belonging to the Otx class of transcription factors (Lamoneirie et al. 1996; Szeto et al. 1996) that is also able to activate α GSU gene expression. Because the expression patterns of P-Lim/Lhx3 and P-OTX/Ptx1 overlap spatially and temporally in Rathke's pouch and thereafter in the developing pituitary gland, it is plausible that these two transcription factors may have a functional relationship (Szeto et al. 1996).

In this work we have used protein interaction screening to identify two highly homologous proteins that interact with the LIM domain of both P-Lim/Lhx3 and Isl-1. The mRNAs encoding these factors can be detected as early as E8.5 during mouse development and are expressed in regions that specifically express LIM homeodomain proteins. Although they cannot activate transcription from the α GSU gene, they are required for a synergistic activation of the α GSU gene by P-Lim/Lhx3 and P-OTX/Ptx1 and are therefore referred to as CLIM-1 and CLIM-2 (cofactor of LIM homeodomain proteins). These results suggest that members of this protein family function, at least in part, by enhancing the transcriptional efficiency of distinct classes of transcription factors by serving as coactivators critical for synergy, thus allowing the LIM and the Otx homeoproteins to be integrated into a larger transcriptional network.

Results

A family of LIM-interacting proteins

To define the factors interacting with the LIM domain of P-Lim/Lhx3, we screened mouse adult pituitary and E14.5 head λ gt11 expression libraries with a radioactively labeled LIM domain of P-Lim. Sequence analysis revealed that all 12 clones that we obtained by this method belonged to a gene family, with at least two members: CLIM-1, isolated from the pituitary library, and CLIM-2, isolated from the embryonic mouse head library. In addition to the P-Lim LIM domain, all clones bound to a radiolabeled protein fragment containing the LIM domain of Isl-1. All clones isolated contained sequences overlapping with the insert of the smallest clone (Fig. 1A), which limits the region in CLIM proteins that is required for the interactions with LIM domains to 109 amino acids. Full-length cDNAs of both transcripts, and one alternatively spliced form of CLIM-1 (CLIM-1b), were obtained by screening an embryonic pituitary library with the inserts of the λ clones originally obtained. Except for a short stretch in their amino termini, CLIM-1a and CLIM-2 are highly homologous throughout their protein sequence, with a 75% overall amino acid identity (Fig. 1B), indicating that CLIM-1 and CLIM-2 define a family of LIM-interacting proteins. Comparison of

Figure 1. Cloning of LIM domain-interacting proteins. (A) Comparison of six representative λ clones obtained in the protein interaction screening with a full-length CLIM-1a cDNA, showing the size and location of the domain on CLIM-1 that is responsible for interaction with the LIM domain. A putative nuclear localization sequence (NLS) is indicated. (B) Amino acid sequence alignment of CLIM-1a, CLIM-1b, and CLIM-2. Identical residues are in bold letters and boxed. A hyphen indicates that this residue is not present. The putative nuclear localization domain is shaded, and an asterisk indicates a Stop codon. The amino-terminal 297 amino acids of CLIM-1a and CLIM-1b are identical. These sequences were deposited in the GenBank database under accession numbers U89487 (CLIM-1a), U89489 (CLIM-1b), and U89488 (CLIM-2).

these two sequences with the sequence data base did not show significant homology to known proteins, and apart from a potential nuclear localization signal (NLS) (shaded in Fig. 1A), no known domains could be identified. However, subsequent to the initial characterization of these cDNAs, two other groups independently reported the sequence of a LIM domain-interacting protein NLI (Jurata et al. 1996) and Ldb1 (Agulnick et al. 1996), both corresponding to the CLIM-2 sequence. Therefore, we refer to the proteins as CLIM-1 and CLIM-2/NLI/Ldb1.

CLIM-1 and CLIM-2/NLI/Ldb1 are transcribed in regions of specific LIM homeodomain expression

The expression patterns of CLIM-1 and CLIM-2/NLI/Ldb1 mRNAs were characterized by RNase protection assays, revealing that transcripts encoding both proteins were expressed in the pituitary gland, brain, testis, kidney, lung, skin, heart, liver, and trigeminal nerve (Fig. 2). The expression levels were not uniform, for example, CLIM-1 mRNA was expressed at highest levels in brain, trigeminal ganglia, and lung (Fig. 2A), whereas CLIM-2/NLI/Ldb1 mRNA showed highest expression levels in the pituitary gland and skin (Fig. 2B). The abundance and the size of the two protected bands detected with the CLIM-1 probe indicated that the alternative spliced transcript encoded by CLIM-1b was expressed at even higher levels than the mRNA of the clone originally obtained, CLIM-1a, and that the ratio of both transcripts was tis-

sue dependent. A second protected band detected with the CLIM-2/NLI/Ldb1 probe suggests that an alternative transcript may also be synthesized for this gene (Fig. 2A,B).

To compare the early expression pattern of CLIM gene family members with P-Lim/Lhx3/m-Lim3 (Seidah et al. 1994; Bach et al. 1995; Zhadanov et al. 1995), Isl-1 (Karls-son et al. 1990), and LH-2 (Xu et al. 1993), we performed whole-mount in situ hybridizations on mouse embryos at stages E8.5, E9.0–9.5, and E11.5. The mRNAs encoding CLIM-1 and CLIM-2/NLI/Ldb1 display overlapping expression patterns with LIM homeoproteins in mouse embryos (Fig. 3). At day E8.5, LIM homeoprotein transcripts were barely detectable, whereas CLIM-1 and CLIM-2/NLI/Ldb1 mRNAs could be readily detected (data not shown). The hybridization patterns of CLIM-1 and CLIM-2 strongly resemble those of the LIM homeodomain genes at E9.0–9.5, with the highest mRNA expression detected in the telencephalon, the mesencephalon, and the metencephalon and with lower levels detected in the neural tube, the limbs, and in the DRG (Fig. 3G,I). At E9.0–9.5 P-Lim was detected in the rostral part of the telencephalon, in the roof of the mesencephalon, in the metencephalon, and in the olfactory epithelium (Fig. 3A); LH-2 transcripts were detected throughout the telencephalon, in the roof of the mesencephalon, in the metencephalon, in the olfactory epithelium, and the fore- and hindlimbs (Fig. 3C). In addition to a hybridization pattern similar to that of P-Lim and LH-2 in the head, specific Isl-1 expression was seen in the DRG and in the neural tube at stage E9.0–9.5 (Fig. 3E). In E11.5 embryos, the overlap in expression of CLIM mRNA with that of LIM homeobox genes was most striking in the developing midbrain and in the hindbrain where P-Lim, LH-2, and Isl-1 are expressed, in the forebrain where LH-2 and Isl-1 are expressed, and in the DRG where Isl-1 is expressed (Fig. 3B,D,F,H,I).

To further analyze the expression patterns of CLIM-1 and CLIM-2/NLI/Ldb1, we carried out in situ hybridizations on mouse embryonic head sections at stage E11.5 to postnatal day (P) 0, using ³⁵S-labeled cRNA probes specific for CLIM-1 and CLIM-2/NLI/Ldb1. Both mRNAs are widely expressed at stage E17 and display a partially overlapping expression pattern in the mouse head (Fig. 4A1,B1). Both transcripts are highest in the developing neocortex, in the hippocampus, in the midbrain, in the medulla (Fig. 4A1,B1 and 4A2,B2), in the trigeminal ganglia (Fig. 4A1,B1, and A3,B3), in the nasal epithelium (Fig. 4A1,B1 and A4,B4), in the submandibular gland (Fig. 4A1,B1, and A5, B5), in the ventral part of the neural tube (Fig. 4A6,B6), and in the DRG (Fig. 4A7,B7). The CLIM-1 probe hybridized in a more restricted fashion than the CLIM-2/NLI/Ldb1 probe, for example, in the neocortex CLIM-1 expression seems restricted to the cortical layers, whereas CLIM-2/NLI/Ldb1 mRNA is also detected closer to the intermediate and ventricular zones. In the olfactory bulb and certain striatal and thalamic brain regions, CLIM-2/NLI/Ldb1 transcripts seemed to be more predominant.

From the expression studies, we conclude that low lev-

Figure 2. RNase protection experiments using RNA from adult mouse tissues. (A) RNase protection using a CLIM-1a-specific probe; (B) RNase protection using a CLIM-2-specific probe; (C) RNase protection using β -actin as probe. The expected bands in each experiment are indicated by arrows.

Figure 3. Comparison of the expression patterns of LIM homeoproteins with CLIM-1 and CLIM-2 by whole-mount in situ hybridizations on mouse embryos. *A, C, E, G, and I* represent mouse embryos at stage E9.0–9.5, and *B, D, F, H, and J* represent mouse embryos at stage E11.5. Embryos in *A* and *B* are hybridized with an P-Lim-specific probe, in *C* and *D* with a LH-2-specific probe, in *E* and *F* with a probe specific for Isl-1, in *G* and *H* with CLIM-1-specific probe, and in *I* and *J* with a CLIM-2-specific probe. The telencephalon (tel), mesencephalon (mes), metencephalon (met), forelimbs (fl), hindlimbs (hl) and dorsal root ganglia (drg) are indicated at stage E9.0–9.5, and at stage E11.5 the forebrain (fb), midbrain (mb), hindbrain (hb) and dorsal root ganglia (drg) are indicated.

Figure 4. In situ hybridization experiments on head sections at mouse embryonic stage 17. (*A*) Hybridizations with a CLIM-1-specific probe; (*B*) hybridizations with a CLIM-2-specific probe. *A1* and *B1* show the whole mouse head [(nc) neocortex; (mb) midbrain; (ml) medulla; (smg) submandibular gland; (tg) trigeminal ganglia; (ne) nasal epithelium; (ob) olfactory bulb; (skn) skin, enlargement of neocortex and hippocampus regions are shown in *A2* and *B2*, the trigeminal ganglia in *A3* and *B3*, the nasal epithelium (*A4/B4*), the submandibular gland (*A5/B5*), the neural tube (*A6/B6*), and DRG (*A7/B7*).

els of CLIM are initially detected broadly in E8.5 embryos before the detection of P-Lim/Lhx3, LH-2, and Isl-1 mRNAs. CLIM mRNA expression then becomes progressively stronger in regions of the developing embryo in which the LIM homeodomain transcripts are abundant. In addition, specific CLIM mRNA expression can be detected in regions of the embryo that do not overlap with expression of LIM homeodomain factors but which overlap with expression of other transcription factors like P-OTX/Ptx1. Interestingly, besides the pituitary gland, transient α GSU expression has been reported in the olfactory placode of mouse embryos (Kendall et al. 1994), overlapping with the expression of CLIM-2/NLI/Ldb1.

The CLIM protein family interacts specifically with P-Lim/Lhx3 and P-OTX/Ptx1

Having shown that the expression of CLIM transcripts overlapped that of LIM homeoproteins and P-OTX, we tested the protein–protein interaction capabilities of P-Lim, CLIM-1, and P-OTX in vitro. Immunoprecipitations were performed, using recombinant expressed proteins and specific antibodies directed against P-Lim and CLIM-1a. 35 S-labeled P-OTX can be immunoprecipitated with the CLIM-1a antibodies upon addition of bacterially expressed, unlabeled CLIM-1a (Fig. 5A). Similarly, 35 S-labeled P-Lim can be immunoprecipitated by CLIM-1a antiserum in the presence of unlabeled CLIM-1a. Both 35 S-labeled P-OTX and CLIM-1a were immunoprecipitable using specific antiserum directed against P-Lim, in the presence of unlabeled P-Lim. These experiments reveal that CLIM-1a can interact with both P-Lim/Lhx3 and P-OTX and that P-Lim can interact with P-OTX.

We then tested whether P-Lim and P-OTX could form stable complexes with CLIM-1 proteins while bound to DNA by performing EMSA experiments with a 32 P-radiolabeled oligonucleotide that contains the P-Lim and P-OTX binding sites of the α GSU promoter (Fig. 5B,C). Full-length P-Lim, but not P- Δ LIM, formed an additional slower migrating complex when CLIM-1a was added to the binding reaction (Fig. 5A). As expected, adding P-Lim antiserum inhibited formation of both complexes, whereas the addition of CLIM-1 antiserum inhibited specifically the slower migrating complex indicating that this complex contains both CLIM-1 and P-Lim. In a similar experiment CLIM-1b also proved capable to interact with P-Lim on DNA (data not shown). We then showed that bacterially expressed P-OTX is also able to associate with CLIM-1a and CLIM-1b in EMSA experiments, demonstrating that this transcription factor is able to complex with CLIM factors on DNA, independently of the presence of LIM homeoproteins (Fig. 5C). When P-Lim, P-OTX, and CLIM-1a were mixed in such an experiment, several even slower migrating complexes appeared, suggesting the formation of complexes consisting of more than two proteins bound to DNA (data not shown). These results demonstrate that protein complexes con-

sisting of at least P-Lim/CLIM-1 and P-OTX/CLIM-1 can form on DNA.

To define the domains of P-Lim that are responsible for interaction with CLIM-1, CLIM-2/NLI/Ldb1, and P-OTX, we tested the binding of 35 S-labeled P-Lim proteins to glutathione S-transferase (GST)–CLIM-1, GST–CLIM-2, and GST–P-OTX proteins (Fig. 6A). P-Lim proteins containing one or both zinc fingers of the LIM domain are capable of interacting with both CLIM and P-OTX. In contrast, a P-Lim protein lacking the entire LIM domain (P- Δ LIM) fails to interact with either CLIM or P-OTX, indicating that the LIM domain is both necessary and sufficient for interaction with these two factors. Although there may be differential contributions of LIM domain 1 and LIM domain 2 for CLIM-1, CLIM-2/NLI/Ldb1, and P-OTX interactions, both are capable of sustaining interactions. The binding of the Isl-1 LIM domain to CLIM-1 was confirmed in this assay (data not shown).

The smallest clone obtained by the initial protein–protein screen corresponds to only 109 amino acids, thus limiting the region that interacts with the LIM domain to amino acids 233–342 of CLIM-1 (indicated in Fig. 6B) and to amino acids 236–344 of CLIM-2/NLI/Ldb1. To further map the protein interaction domain, we tested 35 S-labeled mutant CLIM-1 proteins for their ability to bind to GST P-Lim and GST P-OTX (Fig. 6B). Whereas the CLIM-1a, CLIM-1b and a partial CLIM-1 (amino acids 1–296) protein bind well to P-Lim and P-OTX, the amino acids 1–226 fragment was not able to interact with both proteins (Fig. 6B). These results indicate that the carboxyl terminus of CLIM-1 mediates interactions with both proteins and defines a region of 63 amino acids (amino acids 233–296) as being crucial for efficient interaction with P-Lim and P-OTX.

Similarly, the domain of P-OTX responsible for interactions with P-Lim and CLIM-1 was mapped by analyzing 35 S-labeled P-OTX mutant proteins for their ability to bind to GST P-Lim and GST CLIM-1a (Fig. 6C). The sequence from amino acids 150–281 in the carboxyl terminus of P-OTX was required for efficient interaction with P-Lim and CLIM-1a.

The CLIM proteins enhance the transactivation potential of P-Lim/Lhx3 and are required for a transcriptional synergy between P-Lim/Lhx3 and P-OTX/Ptx1

To determine the cellular location of the CLIM-1a protein, we transfected a cytomegalovirus (CMV) promoter-driven CLIM-1a construct into HeLa cells that was subsequently analyzed by immunostaining with a specific CLIM-1a antiserum. CLIM-1a was clearly localized to the nucleus, consistent with the presence of a putative NLS in its coding region (data not shown).

To begin to evaluate potential effects of the CLIM proteins on the transactivation functions of P-Lim, we co-transfected the –440 mouse α GSU promoter linked to the luciferase gene with plasmids expressing various combinations of P-Lim, P-OTX, and CLIM proteins. It

Figure 5. Interaction of CLIM-1a with P-Lim and P-OTX. (A) CLIM-1a interacts with P-Lim and P-OTX in immunoprecipitation assays. The proteins and the antisera (α) used are indicated. Asterisks indicate in vitro-translated, ³⁵S-labeled proteins. The input ³⁵S-labeled proteins represent 50% of the total amount added for the immunoprecipitations. (B) CLIM-1a binds to P-Lim on DNA. Bacterially expressed CLIM-1a, P-ΔLIM, and P-Lim were used in EMSA experiments, and a double-stranded oligonucleotide containing the P-Lim and P-OTX binding sites was used as probe. For the P-ΔLIM protein, a 1:50 dilution was added to the binding reaction. The protein/DNA complexes are indicated by arrows. (C) CLIM-1 binds to P-OTX on DNA. The same oligonucleotide as in B was used in EMSA experiments with bacterially expressed CLIM-1a, CLIM-1b, and P-OTX. In A, B, and C, all bands originate from the same gels, but some lanes were rearranged for a simpler presentation.

has been reported that 480 bp of the 5'-flanking sequence of the mouse αGSU promoter is sufficient to target expression into Rathke's pouch (Kendall et al. 1994). This promoter region contains at least one LIM homeodomain protein-binding site (-342 to -329) that is recognized by P-Lim and LH-2 (Roberson et al. 1994; Bach et al. 1995) and one Otx-binding site (-390 to -383) which is bound by P-OTX (Szeto et al. 1996). Full-length P-Lim activates this promoter 8-fold, and a P-Lim mutant in which the entire LIM domain had been deleted (P-ΔLIM) activates 25-fold (Fig. 7A). Expression of CLIM-1a alone had no effect on the αGSU promoter, but when cotransfected with full-length P-Lim, the transcriptional activation attained was similar to that observed with P-ΔLIM. In contrast, cotransfection of CLIM-1a with P-ΔLIM showed no additional effect, indicating that the LIM domain is required for the coactivator function of CLIM-1a with P-Lim.

Because we have shown previously that P-Lim and P-OTX can independently activate the αGSU gene (Bach et al. 1995; Szeto et al. 1996), and because CLIM proteins can interact with both P-Lim and P-OTX on DNA, we tested the effect of CLIM proteins on P-OTX (Fig. 7A). P-OTX alone increased the transcription rates of the

αGSU promoter by sevenfold, and cotransfection of CLIM-1 resulted in an additional threefold increase in transactivation. No additive activation effect was observed in cotransfections of full-length P-Lim with P-OTX. However, when P-Lim, P-OTX, and CLIM-1a were cotransfected together, a strongly synergistic activation (134-fold) was measured. Surprisingly, this synergistic effect was also observed when P-ΔLIM was used instead of full-length P-Lim, demonstrating that in this case, the LIM domain was not required. Experiments using either CLIM-1b or CLIM-2/NLI/Ldb1 instead of CLIM-1a gave similar results (data not shown), indicating that the synergistic effect is a general feature of the CLIM factors. Specificity of this effect was documented by cotransfections of CLIM-1a or CLIM-1b with the POU domain transcription factors Pit-1 or Brn-4 with no potentiation observed in the activation of the thyroid-stimulating hormone β-subunit (β-TSH), prolactin, or Pit-1 gene promoter (data not shown). Furthermore, CLIM-1a or CLIM-1b had no effect on the previously described synergy between P-Lim and Pit-1 (data not shown), indicating that the effect of CLIM-1a is both factor- and gene-specific. Together, these results show that CLIM proteins can function as coactivators for two distinct classes of ho-

Figure 6. Mapping of the protein interaction domains in vitro. Full-length and deletion mutant ^{35}S -labeled in vitro-translated proteins (*left*) were tested for their ability to bind to bacterially expressed GST fusion proteins. The relative strengths of interaction were scored from - (background level) to +++ (strong binding). (*Right*) Autoradiographs of representative experiments. The ^{35}S -labeled protein is indicated above the brackets and the GST fusion protein above each lane. Twenty percent of the total ^{35}S -labeled protein input (I) and binding to GST alone (G) are shown. (A) Identification of P-Lim domains that interact with GST-CLIM and GST-P-OTX fusion proteins. (B) Identification of CLIM-1 domains that interact with GST-P-OTX and GST-P-Lim fusion proteins. (C) Identification of P-OTX domains that interact with GST-CLIM-1a and GST-P-Lim fusion proteins.

meodomain transcription factors, represented by P-Lim/Lhx3 and P-OTX/Ptx1, and that they can promote a striking synergy between these two factors.

Discussion

LIM homeodomain transcription factors exert essential functions in the determination of cell lineages during development. However, the specific role of the LIM domain in these factors has been unclear. Several features have been attributed to this structure. In addition to being inhibitory for binding of the holoprotein to DNA (e.g., Sanchez-Garcia et al. 1993), the LIM domain has been shown to be required for synergistic transcriptional activation conferred by LIM homeodomain proteins together with other transcription factors (German et al. 1992; Bach et al. 1995). Furthermore, it has been established that the LIM domain is a structure mediating protein-protein interactions (e.g., Feuerstein et al. 1994; Schmeichel and Beckerle 1994). The isolation of cDNAs encoding the two highly homologous proteins CLIM-1

and CLIM-2/NLI/Ldb1, based on their ability to interact with the LIM domain of P-Lim/Lhx3, has permitted a further clarification of the functions of the LIM domain. The observation that both proteins can interact with the LIM domains of P-Lim/Lhx3 and Isl-1 suggests that members of the CLIM protein family may interact with many or even most of the LIM homeodomain proteins. This hypothesis is supported by the temporal and spatial expression patterns of CLIM-1 and CLIM-2/NLI/Ldb1, which is overlapping with that of LIM homeodomain factors in the mesencephalon, metencephalon, and telencephalon during mouse development. Even at later developmental stages, the regions of highest CLIM-1 and CLIM-2/NLI/Ldb1 gene expression correspond with regions of specific expression of different LIM homeoprotein members. Particularly striking are the parallel expression patterns of Isl-1 (Thor et al. 1991) and CLIM transcripts in the DRG and trigeminal ganglia, where, to our knowledge, Isl-1 is the only expressed LIM homeodomain protein.

LIM domain-dependent CLIM interaction increased

Figure 7. (A) Activation levels of the αGSU promoter linked to the luciferase gene in cotransfections. A schematic of the promoter, including the P-Lim and P-OTX binding sites, is shown at the top. Results are expressed as fold activation, mean ± standard deviation of the mean, compared to activity of the αGSU promoter in the presence of an empty CMV expression plasmid. The combination of cotransfected CMV-driven expression plasmids and the αGSU reporter plasmid are indicated. (B) Model of CLIM action on the αGSU promoter. For synergistic activation all three proteins are required.

the transactivation potential of P-Lim holoprotein by threefold, similar to the activity of LIM domain-deleted protein. These findings are analogous to a study of the *Xenopus* LIM homeodomain factor Xlim1, which is expressed in Spemann's organizer at the gastrula stage (Taira et al. 1992). Xlim1 constructs in which both LIM domains are mutated or deleted, can induce secondary axis formation with associated muscle and neural differentiation, when injected into blastula-stage frog embryos, whereas Xlim1 holoprotein has only a small effect (Taira et al. 1994). *Xenopus* Ldb1 protein proved to enhance the activity of the Xlim1 holoprotein in inducing a secondary axis upon coinjection into blastula-stage frog embryos (Agulnick et al. 1996), arguing for a negative role of the LIM domain.

We have found that CLIM-1 also interacts specifically with a member of the Otx family of transcription factors (P-OTX/Ptx1), consistent with a model where P-Lim, P-OTX, and CLIM-1 can form complexes on DNA. Mem-

bers of the Otx class of the bicoid-related homeobox transcription factors have been proven to play essential roles in vertebrate development. For example, Otx-2 is required for anterior head structures (Acampora et al. 1995; Matsuo et al. 1995; Ang et al. 1996), Prop-1 mutations appear to be responsible for the Ames mouse dwarf phenotype (Sornson et al. 1996) and mutations in the *RIEG* gene, which encodes the solurshin protein, cause Rieger syndrome (Semina et al. 1996). In the homeodomain and its carboxy-terminal half, solurshin shares extensive sequence homology with P-OTX/Ptx1, which is highly expressed in Rathke's pouch and developing pituitary gland (Lamonerie et al. 1996; Szeto et al. 1996). The similar pattern of expression of P-Lim/Lhx3 and P-OTX/Ptx1 throughout the developing pituitary gland (Szeto et al. 1996), the ability of both proteins to activate the αGSU gene in transient transfection assays (Bach et al. 1995; Szeto et al. 1996), and the observations that CLIM mRNA is found in regions of P-OTX expression has led us to consider a functional relationship between the two factors. Whereas cotransfection of P-Lim with P-OTX showed no additive activation effect on the αGSU gene, they produced a strong synergistic activation in the presence of CLIM-1. The fact that the CLIM protein family can also increase the transactivation potential of P-OTX, taken together with the observation that P-OTX and CLIM mRNAs appear to have a similar, overlapping expression pattern in regions such as the submandibular gland, suggests the possibility that CLIM proteins may also modulate functions of members of the P-OTX protein family independently of LIM homeodomain proteins.

Confirming previous reports that the LIM domain is inhibitory for DNA binding of full-length LIM homeoprotein (e.g., Sánchez-García et al. 1993), the deletion of the LIM domain of P-Lim increased binding to its cognate DNA site. Our experiments revealed that CLIM-1a failed to increase binding of P-Lim holoprotein to DNA, as assayed by EMSA with various amounts of P-Lim and CLIM-1. Furthermore, DNase I footprinting experiments using different concentrations of bacterially expressed full-length P-Lim, P-ΔLIM, P-OTX, and CLIM-1 proteins on the αGSU promoter region, which encompasses the binding sites for both proteins, did not reveal any change in the footprint patterns of P-Lim or P-OTX on their respective sites in the presence of CLIM-1 (data not shown). Collectively these findings suggest that release of the inhibitory effect of the LIM domain did not reflect effects on DNA binding by P-Lim.

The results of our EMSA experiments, DNase I footprints, and the observation that CLIM proteins are able to increase the transcriptional activity not only of P-Lim but also of P-OTX (Fig. 7A) argue against a mechanism in which the sole function of CLIM proteins is to increase DNA binding of the associated factor. Furthermore, the synergistic activation observed upon cotransfection of P-ΔLIM, P-OTX, and CLIM-1 cannot be explained by a model in which the only function of the CLIM proteins is to relieve the inhibition of DNA binding conferred by the LIM domain. To explain the function of the CLIM

proteins we prefer a model in which synergy is dependent on the presence of all three proteins in a spatial vicinity, with the CLIM protein associated with P-Lim/Lhx3 and/or P-OTX/Ptx1 (Fig. 7B). Thus, our results suggest that the CLIM proteins are likely to exert at least a component of their biological effects by enhancing transcription and promoting synergism rather than by relieving the inhibition on DNA binding by the LIM domain.

Our in situ hybridization experiments show that both CLIM-1 and CLIM-2/NLI/Ldb1 mRNAs are highly expressed in the ventral part of the developing neural tube (Fig. 4A5,B5). As LIM homeobox genes are expressed in a temporal and spatial gradient along the developing neural tube, subclasses of motor neurons can be distinguished by the expression of a distinct combination of LIM homeobox genes, and a gradient of LIM homeoprotein expression has been reported to be responsible for motor neuron fate specification (Tsuchida et al. 1994; Appel et al. 1995; Pfaff et al. 1996). It is tempting to speculate that the CLIM proteins will play important roles in LIM homeodomain protein-dependent motor-neuron specification. The similar phenotype of mice ($-/-$) for *mLim-1* (Shawlot and Behringer 1995) and *Otx-2* (Acampora et al. 1995; Matsuo et al. 1995; Ang et al. 1996) has prompted these investigators to propose a functional relationship between the LIM and the Otx homeoprotein gene families. The fact that CLIM-encoding mRNAs show a widely overlapping expression pattern with *Otx1* and *Otx2* in the developing mouse brain (Simeone et al. 1992) suggests that the CLIM protein family may play critical roles in the functional relationships of LIM homeoproteins and additional Otx factors.

In summary, we propose that members of the CLIM protein family function as integrator molecules, leading to a synergistic activation of genes expressed in a tissue-specific manner.

Materials and methods

Cloning of CLIM-1 and CLIM-2/NLI/Ldb1

Protein-protein interactions screenings were carried out essentially as described by Kaelin et al. (1992). Briefly, an *XbaI*-*NotI* fragment containing both LIM domains of P-Lim was inserted into the GST bacterial expression vector GST-KGK. The resulting GST fusion protein was radioactively labeled with protein kinase A and [γ - 32 P]ATP (NEN) and used to screen adult mouse pituitary and E14.5 mouse head λ gt11 expression libraries under denaturing/renaturing and nondenaturing conditions. The inserts of positive clones were subcloned into the Bluescript vector pBKS and analyzed by DNA sequencing. Full-length cDNAs were obtained by screening an embryonic pituitary cDNA library (Sornson et al. 1996) using the random primed 32 P-radio-labeled inserts from the clones obtained in the initial protein-protein screening as probes.

RNase protection assays and in situ hybridization

RNase protection assays and in situ hybridizations were performed using cDNA clones (P14 and H5 in Fig. 1A, corresponding to CLIM-1a and CLIM-2, respectively) as templates for T7- and T3-directed cRNA synthesis, generating labeled antisense

probes of 739 and 605 nucleotides, respectively. The RNase protection experiments were done as described previously (Andersen et al. 1993). In situ hybridization of 20- μ m sagittal sections of mouse embryos (E11.5, E15.5, E17, and P0), fixed in 10% buffered formaline, was performed as described previously (Simmons et al. 1990). Whole-mount in situ hybridization studies on mouse embryos E8.5, E9.0–9.5, and E11.5 were carried out essentially as described by Wilkinson (1993). The LIM homeodomain protein antisense probes used for the whole-mount in situ hybridizations were cDNA regions corresponding to amino acids 221–400 of P-Lim (Bach et al. 1995), amino acids 23–426 of LH-2 (Xu et al. 1993), and amino acids 118–348 of Isl-1 (Karlsson et al. 1990).

Antibody generation, immunoprecipitations, and DNA-binding assays

Polyclonal antibodies were raised against the carboxyl termini of the P-Lim protein (amino acids 221–400) and CLIM-1a (amino acids 146–373). Rabbits and guinea pigs were immunized with the respective GST fusion proteins, which were expressed in *Escherichia coli* and partially purified on 10% SDS polyacrylamide gels. Immunoprecipitations were carried out as described previously (Harlow and Lane 1988). The radiolabeled proteins were obtained by in vitro transcription/translation with Promega rabbit reticulocyte lysates and [35 S]methionine and mixed with bacterial expressed proteins and specific antiserum.

Protein-mediated gel shift assays, were carried out as described previously (Ingraham et al. 1988) with the modifications that the binding reaction was carried out on ice and that the gels were run at 4°C. Two microliters of a 1:10 serum dilution was added to the binding reaction and incubated on ice for 10 min. The sequence of the oligonucleotide used in this experiment was 5'-GAAATAATGTAATCCTGAAAATATATCAGGTAC-TTAGCTAATTAATGTGCTACT-3' (sense strand) encompassing the P-Lim and the P-OTX recognition sites on the α GSU promoter.

In vitro protein-protein interaction and transfection assays

For the in vitro protein-protein interaction assays, cDNAs encoding the P-Lim mutants P-Lim LIM Δ C, P-Lim LIM1 Δ C, and P-Lim LIM2 Δ C were synthesized using PCR. The amplified fragments were inserted into the pCITE vector (Novagen). The P-Lim FL, P-Lim LIM1, P-Lim LIM2, P-Lim Δ LIM (P- Δ LIM) and the GST-P-Lim fusion constructs are described in Bach et al. (1995). PCR products of full-length CLIM-1a and CLIM-1b were ligated in-frame into the *XhoI*-*XbaI* sites of the pGEX-KG to yield a GST fusion protein. [35 S]methionine-labeled in vitro-translated protein was used for the in vitro protein-protein interaction assays as described previously (Bach et al. 1995). CLIM-1 carboxy-terminal deletions were obtained by *AvaI* (amino acids 1–226) and *KpnI* (amino acids 1–296) digestion and subsequent in vitro transcription/translations. The amino-terminal deletion mutant (amino acids 146–373) was cloned as an *EcoRI* fragment in-frame into the pCITE vector. The P-OTX carboxy-terminal deletion mutants were obtained by *XhoI* (amino acids 24–271), *SphI* (amino acids 24–237), and *NarI* (amino acids 24–192) digestions and subsequent in vitro transcription/translations. The P-OTX mutants (amino acids 90–315, 150–315, and 24–150) and the GST-P-OTX fusion construct were described previously (Szeto et al. 1996). All in vitro transcription/translations were carried out using the Promega in vitro transcription/translation kit and [35 S]methionine.

In vitro culture and transient transfections of HeLa cells were carried out as described previously (Rhodes et al. 1993). Typi-

cally, 2 μ g of reporter plasmid and 1 μ g of effector plasmid were added as calcium phosphate precipitates per 60-mm plate. The transfected plasmids were α GSU-luciferase reporter plasmid, CMV promoter-driven P-Lim, P-Lim Δ LIM (Bach et al. 1995), P-OTX (Szeto et al. 1996), and CLIM-1a, CLIM-1b, and CLIM-2/NLI/Ldb1. For the full-length CLIM-1a, CLIM-1b, and CLIM-2/NLI/Ldb1 expression plasmids *Hind*III-*Xba*I-digested fragments were cloned into pcDNA3 (Invitrogen).

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A family of LIM domain-associated cofactors confer transcriptional synergism between LIM and Otx homeodomain proteins.

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