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Differential expression of a transcription regulatory factor, the LIM domain only 4 protein Lmo4, in muscle sensory neurons

Hsiao-Huei Chen[1], Joseph W. Yip[1], Alexandre F. R. Stewart[2] and Eric Frank[1,*]

[1]Department of Neurobiology, University of Pittsburgh, Pittsburgh, PA 15261, USA
[2]Department of Medicine, University of Pittsburgh, Pittsburgh, PA 15261, USA
*Author for correspondence (e-mail: efrank@pitt.edu)

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SUMMARY

In the stretch-reflex system, proprioceptive sensory neurons make selective synaptic connections with different subsets of motoneurons, according to the peripheral muscles they supply. To examine the molecular mechanisms that may influence the selection of these synaptic targets, we constructed single-cell cDNA libraries from sensory neurons that innervate antagonist muscles. Differential screening of these libraries identified a transcription regulatory co-factor of the LIM homeodomain proteins, the LIM domain only 4 protein Lmo4, expressed in most adductor but few sartorius sensory neurons. Differential patterns of Lmo4 expression were also seen in sensory neurons supplying three other muscles. A subset of motoneurons also expresses Lmo4 but the pattern of expression is not specific for motor pools. Differential expression of Lmo4 occurs early, as neurons develop their characteristic LIM homeodomain protein expression patterns. Moreover, ablation of limb buds does not block Lmo4 expression, suggesting that an intrinsic program controls the early differential expression of Lmo4. LIM homeodomain proteins are known to regulate several aspects of sensory and motor neuronal development. Our results suggest that Lmo4 may participate in this differentiation by regulating the transcriptional activity of LIM homeodomain proteins.

Key words: Lmo4, LIM-homeodomain proteins, Single cell cDNA libraries, Differential display, Muscle sensory neurons, Neuronal specification, Neuronal differentiation, Chick

INTRODUCTION

A major task for the developing nervous system is to ensure that individual neurons become synaptically connected to each other in an appropriate manner. The stretch-reflex circuit in the spinal cord provides an attractive model to study how neurons establish these specific connections with each other. In this system, sensory neurons supplying muscle spindles (Ia afferents) form strong monosynaptic connections with motoneurons supplying their own muscle but much weaker connections with those supplying other muscles. The specificity of these connections is evident when muscle afferents first make contact with motoneurons and is maintained even in the absence of normal patterns of neural activity (Frank and Wenner, 1993).

To identify genes that may participate in the early specification of Ia sensory neuronal identity, we constructed cDNA libraries from individual Ia sensory neurons that innervate two antagonistic limb muscles, the sartorius and the adductor longus et brevis. Differential screening identified a transcription regulatory factor, LIM domain only 4 protein (Lmo4), that is differentially expressed in sensory neurons supplying different muscles.

Members of the Lmo family of transcriptional regulatory factors lack a DNA-binding domain but contain two protein-protein interaction LIM domains. Lmo proteins compete for NLI (nuclear LIM domain interactor) with LIM homeodomain transcription factors, and thereby regulate formation of LIM homeodomain/NLI complexes and their transcriptional activity (Bach, 2000; Jurata et al., 2000; Rabbitts, 1998). *Drosophila* Lmo plays an important role in wing and peripheral nervous system development by modulating the interaction between the LIM homeodomain protein Apterous and Chip (*Drosophila* homologue of NLI) (Milan and Cohen, 1999; Milan et al., 1998; van Meyel et al., 1999). In addition, Lmo proteins can interact with other transcription factors. In *Xenopus*, the interaction between XLmo3 and the basic helix-loop-helix (HLH) protein HEN1 is involved in the regulation of neurogenesis by activating the expression of *Xenopus* Neurogenin 1 and NeuroD (Bao et al., 2000). Although the expression of *Lmo4* has been reported in mouse motoneurons (Kenny et al., 1998) and DRG neurons (Sugihara et al., 1998) during development, the detailed expression pattern or functional significance of Lmo4 in neurons has not been explored.

We show that Lmo4 is expressed by a subset of sensory and motoneurons shortly after these neurons are postmitotic. Moreover, Lmo4 expression does not require the presence

of limb targets, suggesting that an intrinsic mechanism regulates its early expression in differentiating neurons. Because Lmo4 is co-expressed with LIM homeodomain proteins, it may regulate the functions of LIM proteins during the differentiation of sensory and motoneurons.

MATERIALS AND METHODS

Retrograde labeling and isolation of sensory neurons

After evisceration and exposure of the spinal cord and DRG by ventral laminectomy in E12 (stage 38) chick embryos (Hamburger and Hamilton, 1951), peripheral nerve branches innervating the adductor and sartorius muscles were labeled with a fluorescent marker, lysine-fixable dextran-tetramethylrhodamine or FITC (100 mg/ml in 1% lysolecithin) (Molecular Probes) in cuffs at 32°C overnight in oxygenated MEM (Invitrogen) supplemented with L-glutamine (Invitrogen) and N2 (Invitrogen). The DRG containing the labeled sensory neurons were then removed and dissociated with trypsin/EDTA. Single large labeled cells were visualized using fluorescent optics, and isolated with a suction pipette.

Preparation of single-cell cDNA libraries

Single-cell cDNA libraries were prepared by RT-PCR as described by Dulac and Axel (Dulac and Axel, 1995). The whole cells were transferred into 4 μl cDNA-lysis buffer [1×MMLV buffer, 0.5% NP40 (Sigma), 0.04 μl Prime RNase inhibitor (3′ 5′), 0.04 μl RNAguard (Pharmacia), 0.06 mM dATP, dCTP, dGTP, dTTP, 0.02 OD/ml pd$(T)_{16}$ (Perkin Elmer)] and the cells were lysed at 65°C for 1 minute. The cellular RNA was annealed to Oligo dT at room temperature for 2 minutes and reverse transcribed to synthesize the first strand cDNA with the addition of 0.5 μl of AMV- and MMLV-reverse transcriptases (Invitrogen) (1/1 vol/vol) at 37°C for 15 minutes. The reverse transcriptases were inactivated at 65°C. Poly (A) was added to the first strand cDNA with 10 units of terminal transferase (Roche) in 1×tailing buffer (1×terminal transferase buffer, 0.75 mM dATP) at 37°C for 10 minutes. Terminal transferase was inactivated at 65°C and the cDNA was then amplified in a final 100 μl PCR buffer [1× PCR buffer II, 2.5 mM $MgCl_2$, 0.1 mg/ml BSA, 1 mM dNTP mixture, 0.05% triton X-100, 0.05 μg AL1 primer (5′-ATTGGATCC-AGGCCGCTCTGGACAAAATATGAATTC$(T)_{24}$-3′), 2 μl AmpliTaq (Perkin Elmer)] with 25 cycles of 94°C for 1 minute, 42°C for 2 minutes and 72°C for 6 minutes, with 10 seconds extension time at each cycle. After the first 25 cycles of PCR amplification, an additional 1 μl of AmpliTaq was then added directly into the tubes and another 25 cycles were performed with the same schedule except without the additional extension time. To ensure that only proprioceptive, and not nociceptive, sensory neurons were selected, cDNAs of isolated neurons were screened by PCR for the presence of trkC, a receptor for NT3 expressed in large caliber muscle sensory neurons, and the absence of trkA, a receptor for NGF expressed in many small caliber (both muscle and cutaneous) sensory neurons. The trkC-specific primers were 5′-ATGCAGAGCTGCTGGCAGAGAG-3′ and 5′-CCAAACTGCCTTACAGGTCGTC-3′, and the trkA-specific primers were, 5′-CACGACCTGGTGGTGAAGATTG-3′ and 5′-CTCTCAGCCCAGGATGTCCAGG-3′. To ensure that there was no contamination of Schwann cells during isolation of sensory neurons, cDNAs of isolated neurons were screened by PCR for the absence of myelin basic protein using myelin basic protein-specific primers, 5′-GGCTCTTCTGAATTGCACTG-3′ and 5′-CCACTAT-TACGTTGCCAAG-3. Aliquots of selected adductor and sartorius cDNA stocks were then subjected to Southern blot analysis to determine the accuracy of cDNA representation after PCR amplification. The probes used included genes expressed at high levels (GAPDH and enolase), moderate levels (calcium-ATPase) and low levels (trkC). Of the single cell-cDNA stocks prepared from 99 adductor and sartorius neurons, 5 cDNA stocks that had appropriately amplified levels of marker genes were selected for the final extension step using 1 μl AmpliTaq in 100 μl PCR buffer: 94°C for 5 minutes, 42°C for 5 minutes and 72°C for 30 minutes. The final extended cDNAs were purified and digested with *Eco*RI and cDNA fragments larger than 450 bp were separated on a 1.7% agarose gel and purified. The size-selected cDNA was then packaged using *Eco*RI-digested, dephosphorylated λ Zap II phage arms according to the manufacturer's protocol (Stratagene). The frequency of enolase-positive plaques in each prepared single-cell cDNA library (0.4%) suggested that representation of a given RNA was not biased during the construction of the libraries.

Differential screening of single-cell cDNA libraries

The cDNA libraries were plated at 1000 pfu/plate and duplicate lifts were made of each library. The first library lift was probed with the cDNA probe for the other cell and the second lift with its own cell cDNA probe. Both cDNA probes were prepared by re-amplifying for 10 cycles 1 μl of the original cell cDNAs in 50 μl PCR buffer with the AL1 primer, but cold dCTP was replaced with 100 μCi of [P]-dCTP (3000 μCi/mmole) as described by Dulac and Axel (Dulac and Axel, 1995). Positive plaques in duplicate lifts were compared and clones that showed differential expression were isolated. After cross-screening 15,000 recombinant phages prepared from single-cell cDNA stocks of three adductor and two sartorius neurons, the inserts of 95 candidate plaques were amplified using T3 and T7 primers and cell-specific expression was confirmed using cDNA probes from the original adductor and sartorius sensory neurons in Southern blots. Of those, five clones were sequenced and chosen for further PCR/Southern blot analysis with single-cell cDNA stocks prepared from 17 additional adductor and sartorius cells using clone-specific primers. Lmo4 was confirmed to be differentially expressed in adductor but not sartorius sensory neurons by PCR using Lmo4-specific primers, 5′-GTTCATC-ACAGATGGATCCCCATG-3′ and 5′-GCCATGGGAAGTAGCA-ACATTAGG-3′ (see Fig. 1).

Isolation of chick *Lmo4* gene

The full-length coding sequence of *Lmo4* was isolated from a random-primed E9 chick brain cDNA library (a gift from Dr W. Halfter, University of Pittsburgh, PA). DNA was sequenced on both strands at the University of Pittsburgh Sequencing Core Facility (GenBank Accession Number, AF532926).

Immunohistochemistry

For cell counts of retrograde FITC-labeled sensory and motoneurons, embryos were fixed with 4% paraformaldehyde for 2-3 hours, washed in PBS and sectioned at 12 μm. Lmo4 protein was visualized using an Lmo4-specific goat polyclonal antibody (Lmo4-c15, Santa Cruz Biotechnology) detected with a Cy3-conjugated donkey anti-goat secondary antibody (Jackson Laboratories). In other sections, Cy3- and FITC-conjugated secondary antibodies were used for dual immunohistochemistry.

Monoclonal antibodies specific for Isl1 and Isl2 (4D5), Lim1 and Lim2 (4F2), and Lim3 (67.4E12) were obtained from the Hybridoma Bank (University of Iowa). A monoclonal antibody specific for ER81 (5B10) and a rabbit antibody specific for chick Pea3 (C115) were generously provided by Dr T. M. Jessell (Columbia University).

Limb ablation

Unilateral ablation of hindlimb bud precursors including the ectoderm and underlying lateral plate mesoderm was performed in seven embryos at stages 15 or 18. Embryos developed to stages 27-29 and were then processed as described above.

RESULTS

Differential screening of single-cell cDNA libraries from muscle sensory neurons

Many studies of neuronal specification in the spinal cord have taken advantage of the topographic organization of motoneurons into specific muscle pools (Hollyday, 1980a; Hollyday, 1980b; Landmesser, 1978) to delineate their transcriptional program. Both LIM homeodomain transcription factors and members of the ETS family of zinc-finger transcription factors have been implicated in establishing motoneuron pool identity (Lin et al., 1998; Tsuchida et al., 1994). However, mechanisms that underlie the specification of Ia sensory neuronal identity are still poorly understood.

Unlike motoneurons that are topograpically organized in the spinal cord, functionally distinct sensory neurons that project to different muscles are dispersed throughout the DRG. This dispersal has made it difficult to identify pool-specific marker genes in sensory neurons. To overcome this problem, we combined the method of differential screening of single-cell cDNA libraries used successfully in identifying pheromone receptors (Dulac and Axel, 1995) with retrograde labeling of sensory neurons from identified muscle nerves. Using this approach, we screened for genes that are differentially expressed by proprioceptive sensory neurons (Ia afferents) innervating two different and antagonistic limb muscles, the sartorius and the adductor. These muscles were chosen because the Ia afferents supplying them make different sets of synaptic connections with motoneurons. Sartorius afferents make strong connections with sartorius motoneurons but virtually none with adductor motoneurons, while the converse is true for Ia afferents supplying the adductor muscle (Mendelson and Frank, 1991). In addition, sartorius and adductor motoneurons are located at a similar rostrocaudal location in the cord, so sensory afferents must distinguish between correct and incorrect synaptic partners within the same target region (Mendelson and Frank, 1991). Differences in gene expression between these two groups of proprioceptive sensory neurons therefore are likely to include genes that reflect or influence the selection of their peripheral and central targets.

By differentially screening cDNA libraries of adductor

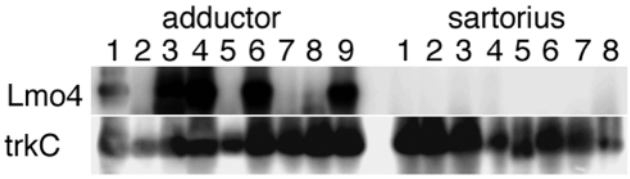

Fig. 1. Identification of *Lmo4* as a differentially expressed transcript in adductor but not sartorius sensory neurons by single-cell RT-PCR. PCR using *Lmo4*-specific oligonucleotide primers amplified a 544 bp fragment in single cell cDNAs derived from five out of nine adductor neurons but none out of eight sartorius neurons at stage 39. The PCR product was detected by Southern hybridization using the *Lmo4* cDNA. trkC cDNA was PCR-amplified as a positive control from all cells.

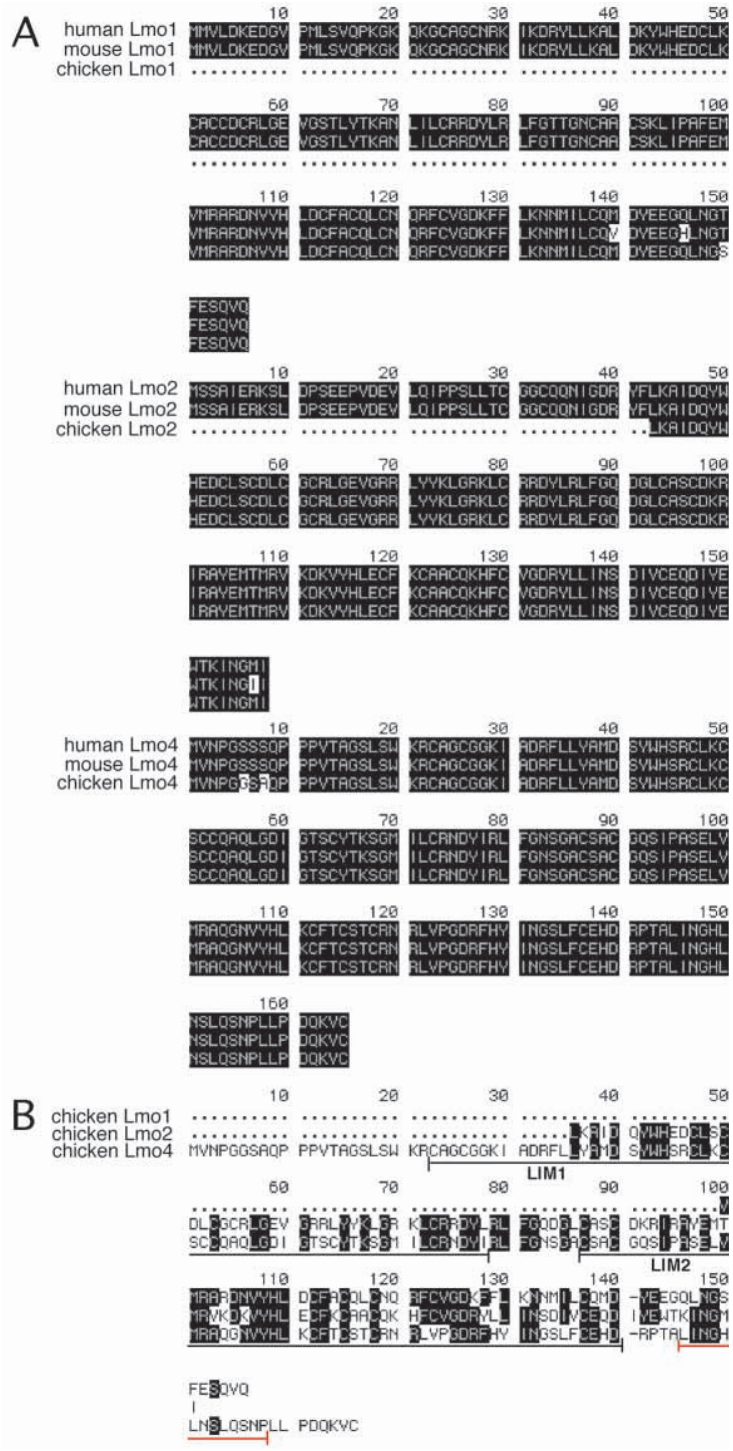

Fig. 2. Sequence analysis of Lmo proteins from human, mouse and chicken sequences. (A) The non-mouse non-human expressed sequence tag (EST) database was searched with mouse amino acid sequences using the tBLASTn algorithm to identify cDNA sequences of chicken Lmo1 (GenBank Accession Number, AL587905) and Lmo2 (GenBank Accession Number, BI067394). Sequences are aligned to show the high degree of homology within Lmo family members of different species. Dots indicate missing sequence. (B) Sequence alignment of chicken Lmo1, Lmo2 and Lmo4 reveals high divergence overall; only three out of 13 amino acids are shared between Lmo1, Lmo2 and Lmo4 in the region (amino acids 145-157, underlined in red) used to make the Lmo4-specific c15 antibody. Lim1 and Lim2 domains are underlined in black.

and sartorius sensory neurons of stage 39 chick embryos, a stage when Ia afferents are making synaptic connections with motoneurons (Lee et al., 1988), we identified a transcript of the chick homolog of the LIM domain only 4 protein (Lmo4) that was present in most adductor but not sartorius single-cell cDNA libraries. The adductor-specific expression of *Lmo4* was further confirmed by Southern blot analysis of a second series of single-cell cDNA stocks by PCR using *Lmo4*-specific primers. *Lmo4* was detected in five out of nine adductor and none of eight sartorius cells (Fig. 1).

The *Lmo4* cDNA was used as a probe to isolate full-length clones from a random primed E9 chick brain cDNA library that contained larger inserts than those in our single-cell cDNA libraries. The full-length Lmo4 clone encodes a peptide of 165 amino acids that contains two LIM domains (Fig. 2). The Lmo4 homolog in chick is 80% conserved at the DNA sequence level with human and mouse Lmo4. At the amino acid level, however, it differs from these proteins by only two amino acids (6 and 8). Such a high degree of similarity suggests a highly conserved function in birds and mammals.

Lmo4 is expressed in subsets of sensory afferents

The pattern of Lmo4 expression in the DRG was determined both by RNA in situ hybridization and by immunohistochemistry using the Lmo4-c15 antibody in stages 35 and 39 chick embryos. The Lmo4-c15 polyclonal antibody was generated from a C-terminal peptide of human Lmo4 located between amino acids 145 and 157 (Santa Cruz Biotechnology), a region where chicken, mouse and human Lmo4 are identical (Fig. 2A). The antibody should therefore recognize chicken Lmo4. Direct evidence on this point was obtained by immunohistochemistry and western blot analysis of 293 cells transiently infected with a full-length chicken Lmo4 expression construct. Only infected cells were recognized by the antibody (data not shown). cDNA sequences encoding chicken Lmo1 and 2 were identified in the EST database and aligned to the human and mouse homologs (Fig. 2A). It is unlikely that the Lmo4-c15 antibody crossreacts with these proteins in chickens. Lmo4-c15 does not recognize human Lmo family members except Lmo4 in western blots (Santa Cruz Biotechnology), and the partial sequences for chicken Lmo1 and Lmo2 are virtually identical to their human homologs (Fig. 2A). Furthermore, the C-terminal sequences of chicken Lmo1, Lmo2 and Lmo4 are highly divergent. Only three out of 13 amino acids in the region used to make the antibody are shared among these family members (underlined in red in Fig. 2B), making it improbable that the antibody recognizes chicken Lmo1 or Lmo2. Finally, the patterns of immunoreactivity to Lmo4-c15 and in situ labeling of Lmo4 mRNA are highly similar in the spinal cord and DRG, including the extent of labeling of specific sensory and motor pools (see below and data not shown).

Lmo4 protein is mainly expressed in the ventrolateral (VL) part of the DRG, where most large diameter, trkC-positive, muscle sensory cells are located. Out of all the trkC-positive neurons, 83% were Lmo4 positive. By contrast, very few of the dorsomedially (DM) located small diameter, trkA-positive,

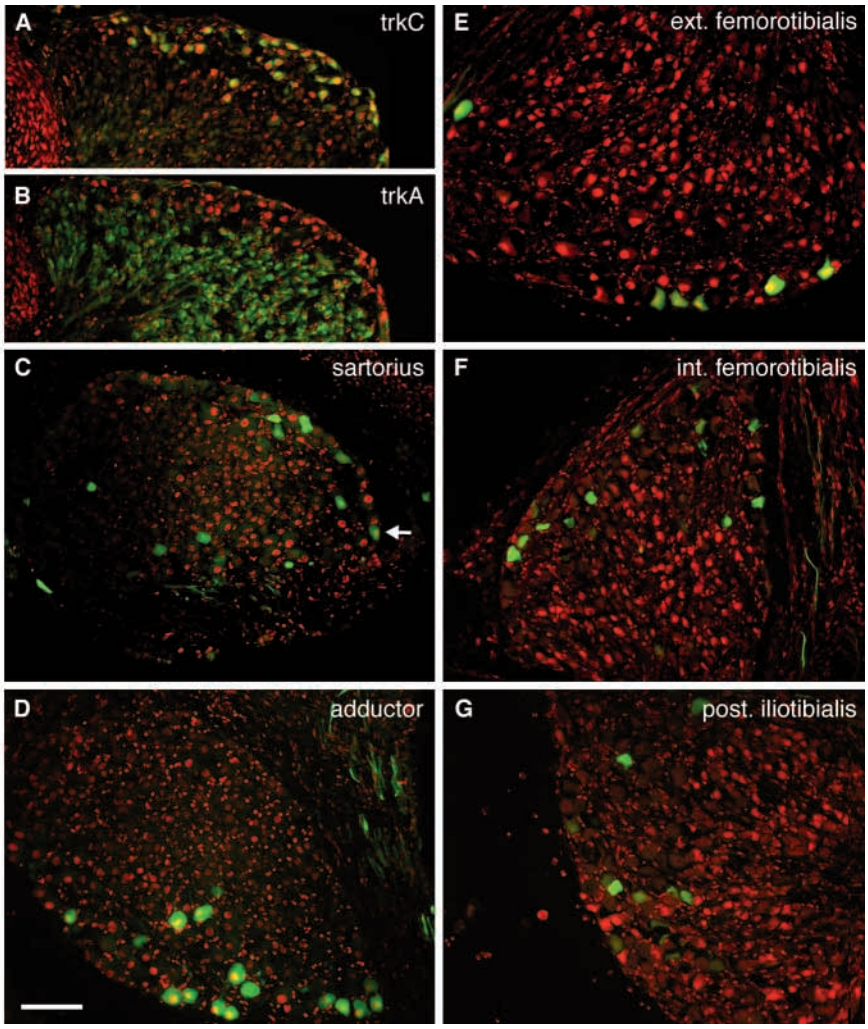

Fig. 3. Lmo4 is expressed in trkC-positive proprioceptive sensory neurons in muscle-pool-specific patterns. Lmo4 expression in nuclei of DRG sensory afferents was detected with an Lmo4-specific antibody (red in all panels). At stage 35, trkC- and trkA-specific antibodies stain (green) cytoplasm of large-diameter trkC-positive proprioceptive sensory neurons located ventrolaterally (upper right-hand corner in A) and small-diameter trkA-positive cutaneous sensory neurons located dorsomedially (lower left-hand corner in B) in the DRG, respectively. Most (83%) trkC-positive sensory neurons are Lmo4 positive, whereas almost none of the trkA-positive sensory neurons express Lmo4. (C-G) Lmo4 expression in sensory neurons supplying individual muscles was determined by retrograde labeling with green fluorescent dye at stage 39. Lmo4 is expressed in most adductor (79%, D) and external femorotibialis (73%, E) sensory neurons, but few sartorius sensory neurons are Lmo4-positive (13%, C) (one Lmo4-positive cell is indicated with arrow). Sensory neurons supplying the internal femorotibialis (F) and posterior iliotibialis (G) muscles are Lmo4 negative. Scale bar: 150 μm.

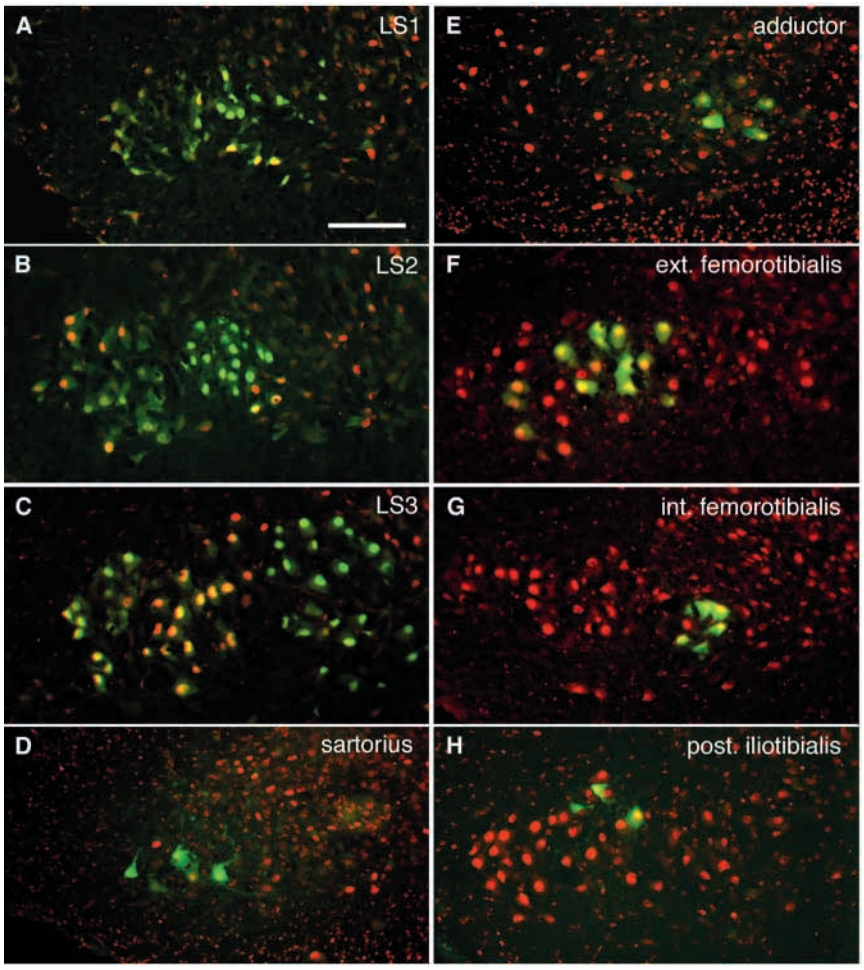

Fig. 4. Lmo4 is expressed in subsets of LMC motoneurons in the lumbosacral spinal cord (stage 39). (A-C) Isl1/Isl2 (green) is expressed in all LMC neurons, whereas Lmo4 (red) is expressed in some of these cells. (D-H) Lmo4 expression in motoneurons supplying individual muscles was determined by retrogradely labeling with green fluorescent dye at stage 39. Lmo4 (red) is expressed in most adductor (84%, E), external femorotibialis (92%, F) and posterior iliotibialis (95%, H) motoneurons. Few sartorius motoneurons express Lmo4 (15%, D) and about half of the internal femorotibialis motoneurons are Lmo4 positive (40%, G). Only the LMC is shown; lateral is towards the left. Scale bar: 100 μm.

cutaneous sensory neurons express Lmo4 (Fig. 3A,B). Similar expression patterns were seen using in situ hybridization (data not shown). To investigate the profile of Lmo4 expression by sensory neurons supplying individual muscles, we combined retrograde labeling of identified peripheral nerves with immunohistochemistry or in situ hybridization for *Lmo4* mRNA.

The difference in Lmo4 expression seen in Southern blots of single-cell cDNAs was confirmed histologically on sections of labeled neurons. Nearly 80% of sensory neurons supplying the adductor muscle are Lmo4 positive compared with only 13% of sartorius sensory neurons (Fig. 3C,D; Table 1). Similarly, 70% (153/220) of retrogradely labeled adductor neurons express *Lmo4* mRNA versus 10% (nine out of 91) of sartorius sensory neurons (data not shown). To determine if Lmo4 identifies only those afferents supplying ventral (including adductor) but not dorsal (including sartorius) limb muscles, we also examined sensory neurons projecting to other dorsal limb muscles, including iliotibialis and the internal and external heads of femorotibialis. Seventy-three percent of external femorotibialis neurons are Lmo4 positive, whereas only 2-3% of internal femorotibialis and posterior iliotibialis neurons are Lmo4 positive, demonstrating that Lmo4 expression is not restricted simply according to the dorsal/ventral innervation of the limb (Fig. 3E-G; Table 1).

In addition to its expression in sensory neurons, Lmo4 is expressed in satellite and Schwann cells in the DRG and spinal roots. As in neurons, the protein is restricted to nuclei, visible throughout the DRG (small profiles in Fig. 3C-G) and in spinal roots (right side of Fig. 3F). Lmo4 is also expressed in small, non-neuronal cells in the ventral white matter of the spinal cord (Fig. 4D,E). Expression of Lmo4 mRNA in non-neuronal cells in murine DRGs has been reported previously (Sugihara et al., 1998).

Lmo4 is expressed in subsets of motoneurons

To determine whether Lmo4 is also differentially expressed in motoneurons supplying limb muscles, we examined its expression in the cord at lumbosacral (LS) levels LS1-LS3 at stage 39. Within LS1, most motoneurons (identified by Isl1/Isl2 expression) within the lateral motor column (LMC) do not express Lmo4. But within LS2 and LS3, Lmo4 is expressed in approximately half of LMC motoneurons (Fig. 4A-C). To determine if the expression is specific for motor pools, we labeled individual pools using retrograde fills from muscle nerves (Fig. 4D-H; Table 1). Several motor pools have the same fraction of Lmo4-positive neurons as the corresponding sensory pools. For example, 80-90% of adductor and external femorotibialis motoneurons are Lmo4 positive, compared with 75-80% for the corresponding sensory neurons. By contrast, only 15% of sartorius motoneurons express Lmo4, compared with 13% for sartorius sensory neurons. Expression of Lmo4 mRNA, determined by in situ hybridization, is similar for each of these motor pools (91%, 92% and 10%, respectively; data not shown). Sensory and motoneurons supplying the same muscle do not

Table 1. Lmo4 expression in lumbosacral DRG sensory and motoneurons (LS1-5) at stage 39

Labeled nerve	% of labeled SN	% of labeled MN
Sartorius	13% (19/148)	15% (22/143)
Adductor	79% (228/289)	84% (81/97)
Ext. femorotibialis	73% (249/340)	92% (205/223)
Int. femorotibialis	2% (5/262)	40% (30/74)
Post. iliotibialis	3% (14/491)	95% (55/58)

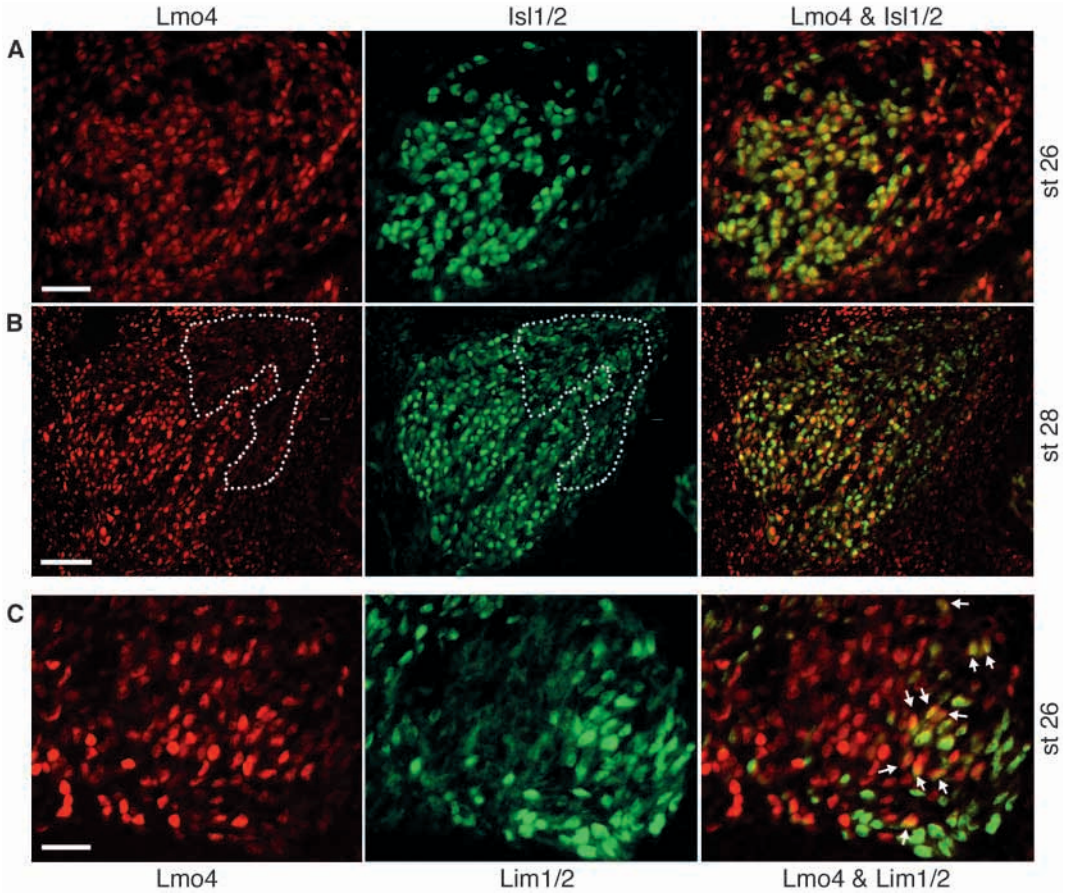

Fig. 5. Co-expression of Lmo4 and LIM homeodomain proteins by neurons at earlier developmental stages. Localization of Lmo4 (red) and the LIM homeodomain proteins Isl1/Isl2 and Lim1/Lim2 (green) was determined by double-label immunohistochemistry in DRG sensory neurons (A,B) and in lumbosacral spinal motoneurons (C). At stage 26, Lmo4 is co-expressed with Isl1/Isl2 in DRG neurons (A). At stage 28, Lmo4 is excluded from DM cutaneous sensory neurons (B, dotted outline indicates the major region of non-overlap of Lmo4 and Isl1/Isl2). (C) At stage 26, Lmo4 is expressed in a subset of Lim1/Lim2-positive (l) LMC motoneurons at LS2 (arrows). The dorsomedial region of DRGs is located in the upper right in A,B. Lateral is towards the right in C. Scale bars: 25 μm in A,C; 50 μm in B.

always share similar Lmo4 expression patterns, however. Virtually all posterior iliotibialis motoneurons express Lmo4, while only 2% of the sensory pool is Lmo4 positive. Furthermore, the expression of Lmo4 is not strictly organized with respect to motor pools; an intermediate fraction (40%) of internal femorotibialis motoneurons is Lmo4 positive.

Neurons with common expression patterns of LIM homeodomain proteins can differ in their Lmo4 expression during development.

LIM homeodomain transcription factors are expressed early during sensory and motoneuron development and are important for their functions. Null mutation of Isl1 prevents the development of sensory and motor neurons (Pfaff et al., 1996). In the spinal cord, combinatorial expression of the LIM homeodomain proteins Isl1, Isl2 and Lim1 defines subclasses of motoneurons that segregate into columns and select distinct projection pathways in the periphery (Kania et al., 2000; Tsuchida et al., 1994). Co-expression of Lmo4 can modulate the transcriptional activity of LIM proteins and may thereby influence neuronal differentiation.

The observation that Lmo4 is expressed in only some sensory pools at stage 39 raised the possibility that Lmo4 might be differentially expressed in sensory neurons at earlier developmental stages as well. At stage 26, when muscle sensory neurons are establishing their first peripheral projections and before many cutaneous (DM) neurons are born, Lmo4 is expressed in many Isl1/2-positive muscle sensory neurons (Fig. 5A). By contrast, Lmo4 expression is excluded from DM neurons from the outset, even before they develop a trkA-positive/trkC-negative phenotype (area outlined by dots in Fig. 5B). By stage 27, when Pea3 expression begins in sensory neurons, Lmo4 is not expressed in a subset of Pea3-positive cells (14%, Fig. 6A). Pea3 is expressed only in proprioceptive sensory neurons (Lin et al., 1998), so the expression of Lmo4 in some but not all of these cells suggests that, even at this stage, only a subset of muscle sensory neurons are Lmo4 positive. Similarly, at stage 35, when trkC provides a good marker for proprioceptive neurons, 17% of trkC-positive cells are Lmo4 negative (Fig. 6B). Thus, Lmo4 has a restricted expression pattern in sensory neurons at a time when they are making their peripheral and central connections.

Differential co-expression of Lmo4 with LIM proteins was also detected in motoneurons before they develop their distinctive ETS phenotype. At stage 26, some neurons in the lateral LMC are Lmo4 positive (arrows in Fig. 5C) while others are Lmo4 negative, even though all are Lim1/2 positive. As motoneurons located in the same subdivision and sharing the same expression profile of LIM homeodomain proteins innervate several different limb muscles, differential co-expression of Lmo4 might serve to direct otherwise similar motoneurons along different developmental pathways.

Lmo4 expression does not predict expression of ER81 or Pea3

The ETS proteins ER81 and Pea3 are expressed in subsets of sensory and motoneurons in a pool-specific manner. We

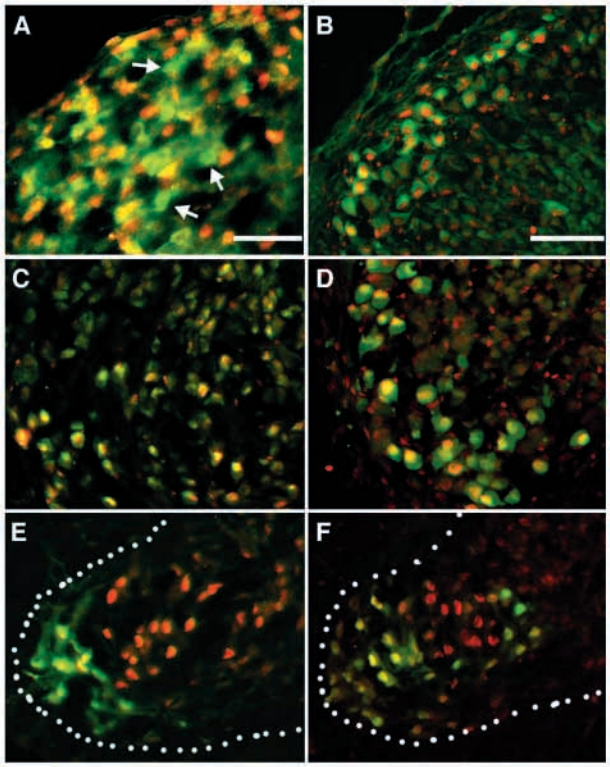

Fig. 6. Comparison of Lmo4 (red in all panels) with the ETS gene Pea3 (green in A,C,E) and ER81 (green in D,F) in sensory and motoneurons. (A) At stage 27, most Pea3-positive (green) sensory neurons are Lmo4 positive (yellow cells), but a small subset (arrows) is Lmo4 negative. (B) By stage 35, when trkC expression (green) serves as a good marker for muscle proprioceptive sensory neurons, a small proportion of trkC-positive cells remain Lmo4 negative. At this stage, Lmo4 is expressed in many, but not all, Pea3-positive (C) and ER81-positive(D) sensory neurons. In the LS2 spinal cord, Lmo4 is expressed in many, but not all, Pea3-positive (E) and ER81-positive (F) LMC motoneurons. See also Table 2. Scale bars: 25 μm in A; 100 μm in B-F.

therefore determined whether Lmo4 expression was correlated with either of these ETS family members. Expression in some sensory pools did match that of ER81. The adductor and external femorotibialis pools, which have high levels of Lmo4 expression, also express ER81, whereas sartorius and internal femorotibialis sensory neurons, most of which do not express Lmo4, are also not ER81 positive. But the correlation for all sensory neurons is not strong. Only 65% of ER81-positive sensory neurons are Lmo4 positive at stage 35. The fraction of ER81-positive cells that are also Lmo4-positive is similar in each of LS1 through LS4. Lmo4 expression also does not correlate with the presence or absence of Pea3; 71% of Pea3 sensory neurons are Lmo4 positive (Fig. 6C,D; Table 2)

A similar situation is found for Lmo4 and ETS expression in motoneurons. The expression of Lmo4 and ER81 is correlated in the adductor, sartorius and external femorotibilas motor pools. But posterior iliotibialis motoneurons, which are Lmo4 positive, are located mainly in LS4 where there is virtually no expression of ER81 (Lin et al., 1998). In addition, internal femorotibialis motoneurons are also ER81 negative,

Table 2. Co-expression of Lmo4 and other muscle sensory neuron markers in lumbosacral DRG proprioceptive sensory neurons (LS1-4) during development

Stage	% of Pea3-positive cells	% of ER81-positive cells	% of trkC-positive cells
27	86% (132/154)	NA†	NA‡
35	71% (63/159)	65% (171/317)	83% (235/280)

†At stage 27, sensory neurons do not yet express ER81.
‡TrkC is expressed in both proprioceptive sensory neurons and trkA-positive cutaneous sensory neurons at stage 27; therefore, trkC is not an appropriate marker at this stage.
NA, not applicable.

but 40% of them express Lmo4 (Table 2). A substantial portion of Pea3-positive motoneurons, which do not express ER81, also express Lmo4 (Fig. 6E,F; Table 2). These results suggest that although the expression of Lmo4 precedes that of ETS genes in sensory and motor neurons, it does not predict the later expression of these genes.

Lmo4 expression does not require signals from the limb

Some aspects of sensory neuronal phenotype, such as the specification of muscle versus cutaneous targets, are determined before peripheral projections are established (Honig et al., 1998; Ma et al., 1999; Oakley et al., 2000). Motor neuronal phenotype is also largely specified before target innervation (Lance-Jones and Landmesser, 1980). For both sensory and motor neurons, expression of LIM homeodomain proteins occurs shortly after exit from the cell cycle and is not blocked by removal of their peripheral targets (Ensini et al., 1998; Lin et al., 1998; Sockanathan and Jessell, 1998). By contrast, the pool-specific factors ER81 and Pea3 that are expressed at later times require peripheral targets, as limb ablation blocks their expression. Lmo4 is expressed relatively early, as are the LIM proteins, yet its expression in sensory neurons is correlated with peripheral target identity, as are the ETS proteins. It was therefore of interest to learn if the expression of Lmo4 required peripheral targets.

To investigate the influence of peripheral targets on Lmo4 expression, we unilaterally ablated hindlimb buds at stage 15. No obvious difference in Lmo4 protein or mRNA expression was seen in DRG after ablation at either stage (Fig. 7A; data not shown). Lmo4-positive sensory neurons were restricted to the large cell population and were located in the ventrolateral portion of the DRG as in normal embryos. By contrast, Pea3 expression was reduced, as reported previously (Lin et al., 1998) (Fig. 7B).

The observed loss of Pea3 expression is complicated by the fact that limb bud ablation induces an early peak of cell death in DRG (Caldero et al., 1998). This peak occurs at the stage when we assessed Lmo4 and Pea3 expression after ablation, and it is confined largely to the VL (proprioceptive) population. Early death of VL neurons is unlikely to influence our conclusions about Lmo4 expression, however, because there is no obvious reduction in expression. Furthermore, the persistence of Lmo4 expression provides additional support for the conclusion by Lin and co-workers that loss of Pea3 expression is not due to cell death (Lin et al., 1998). Most Pea3-positive neurons co-express Lmo4 at stage 27, so the

persistence of normal numbers of Lmo4-positive cells implies that the cells have not died.

The effect of limb bud deletion on Lmo4 and Pea3 expression in motoneurons is similar to that in sensory neurons. The number and location of Lmo4-positive motoneurons are unchanged after ablation. Pea3 expression in motoneurons, however, is largely abolished (Fig. 7D,E) (Lin et al., 1998). Thus, for both sensory and motor neurons, expression of Lmo4 is determined intrinsically; it does not require peripheral targets.

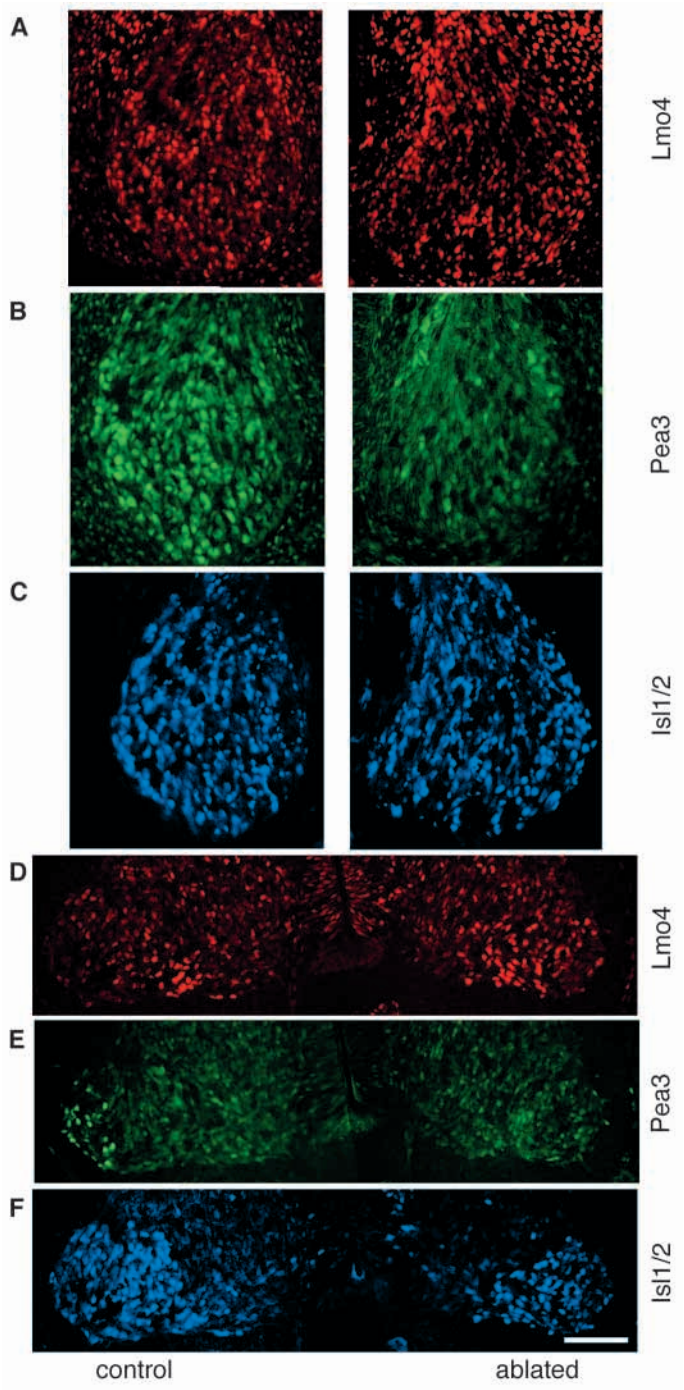

Fig. 7. Limb ablation does not alter Lmo4 expression. Limb ablations were performed unilaterally at stage 15 and analyzed at stage 28. Lmo4 expression appeared unchanged in DRG (A) and in the spinal cord at LS2 (D) after limb ablation. By contrast, Pea3 expression was lost after limb ablation (B,E). (C,F) Sections adjacent to the dual-stained A,B,D,E sections were stained for Isl1/Isl2 expression to estimate numbers of surviving neurons. Scale bar: 85 μm.

DISCUSSION

We have identified the chick homolog of the mammalian *Lmo4* gene and shown that Lmo4 is expressed in a subset of sensory and motoneurons. Although Lmo4 is expressed in the majority of muscle sensory (trkC-positive) neurons, it is excluded from sensory neurons projecting to certain muscles. Unlike the motor pool-specific ETS family of transcription factors, Lmo4 expression begins shortly after these neurons exit from the cell cycle, and does not require peripheral signals from the limb. The presence of two LIM interaction domains in the Lmo4 protein suggests that in combination with other transcription factors, Lmo4 may modulate LIM homeodomain transcriptional activities as an intrinsic signal that contributes to the specification of sensory and motor neurons.

Lmo4 expression in developing sensory neurons

The results described here show that Lmo4 is expressed in muscle pool-specific patterns in sensory neurons. We initially isolated *Lmo4* because of its differential expression in sensory neurons supplying the adductor versus sartorius muscles. In addition to sartorius sensory neurons, neurons supplying the internal femorotibialis and posterior iliotibialis muscles are also Lmo4 negative, while most sensory neurons projecting to external femorotibialis are Lmo4-positive. Despite the fact that Lmo4 is expressed in most (83%) trkC-positive sensory neurons, its absence from those neurons supplying specific muscles suggests that it may contribute to muscle pool specificity of these cells.

Lmo4 is already expressed by stage 26, when sensory neurons are establishing their first peripheral projections and before many cutaneous (DM) neurons are born. The exclusion of Lmo4 from DM neurons, clearly visible at stage 35 after these cells have developed their distinctive trkA-positive phenotype, is apparent by stage 28. Even within the population of muscle sensory neurons, early Lmo4 expression appears to be selective. Based on co-expression with Pea3, Lmo4 is selective for a subset of muscle sensory neurons by stage 27. A similar fraction of neurons co-express trkC and Lmo4 at stage 35. Although there is no direct evidence that Pea3-positive sensory neurons at stage 27 represent the same population as trkC-positive sensory neurons at stage 35, the similarity in fractions of Lmo4-negative neurons at these two stages suggests that Lmo4 expression may be determined already by stage 27, before most muscle sensory neurons have reached their peripheral targets.

A separate line of evidence supporting the idea that Lmo4 expression does not require peripheral targets is provided by the limb ablation experiments. When hind limb buds are removed at stage 15, there is still extensive expression of Lmo4 within the DRG at stage 28. The persistence of Lmo4 expression implies that Lmo4, like Isl1/Isl2, is an intrinsic signaling factor in sensory neurons. Instead of decreasing expression, limb ablation might actually increase expression of

Lmo4. Relatively few proprioceptive muscle sensory neurons (~15%) normally do not express Lmo4, so expression by all proprioceptive neurons would only result in a small fractional increase, which could be difficult to observe. According to this interpretation, certain peripheral targets, such as the sartorius, internal femorotibialis and posterior iliotibialis muscles, could modulate the phenotype of their sensory innervation by inhibiting expression of Lmo4. Muscle targets of proprioceptive sensory neurons are believed to specify the synapses these neurons make within the spinal cord (Frank and Wenner, 1993), and inhibition of Lmo4 expression could then be one mechanism contributing to this specification.

The only other proteins known to be expressed in sensory neurons in a pool-specific fashion are the ETS transcription factors ER81 and Pea3. Like Lmo4, ER81 is expressed in the majority of sensory neurons supplying the adductor and external femorotibialis muscles, but in few sartorius or internal femorotibialis sensory neurons (Lin et al., 1998). Because Lmo4 is expressed before sensory neurons attain their definitive ETS phenotype, these results suggest that Lmo4 expression might predict the later expression of ER81. In this regard, it is interesting that Lmo4 expression does not require that sensory neurons have peripheral targets. By contrast, the definitive ETS phenotype of sensory neurons is expressed only after they contract their peripheral targets, and ETS gene expression is dependent on these targets. A possible scenario is that expression of Lmo4 could be permissive for ER81, but the ultimate expression of ER81 also depends on contact with the appropriate peripheral target. According to this idea, only a fraction of Lmo4-positive neurons will also be ER81 positive, which would explain why many sensory neurons in LS1 are Lmo4 positive but few express ER81. A weakness of this idea, however, is that many ER81-positive neurons do not express Lmo4, at least by stage 35. Throughout LS1 to LS4, 35% of ER81-positive neurons are Lmo4 negative, and the fraction of Lmo4-negative/ER81-positive cells is similar in each of these segmental ganglia. It is therefore unlikely that Lmo4 expression plays a major role in determining the ultimate ETS phenotype of developing sensory neurons.

Lmo4 expression in developing motoneurons

Many functional subsets of motoneurons within the LMC can be defined by co-expression of LIM homeodomain proteins and ETS proteins (Lin et al., 1998). The differential expression of LIM homeodomain proteins by medial and lateral LMC neurons is established early, soon after neurons exit the cell cycle and migrate to the LMC (Tsuchida et al., 1994; Ensini et al., 1998; Sockanathan and Jessell, 1998). As early as stage 26, when motor axons are making selective projections to their peripheral targets, Lmo4 is already expressed in subsets of motoneurons that otherwise share the same pattern of LIM and Isl expression. As Lmo4 could modify the transcriptional activity of LIM homeodomain proteins, differential expression of Lmo4 in motoneurons may serve to direct otherwise similar motoneurons along different developmental pathways. Like LIM proteins that are important in the early determination of motor pool identity, expression of Lmo4 in motoneurons does not require signals from limb tissue. It is possible that early differential expression of Lmo4 seen in motoneurons at stage 26 may just reflect their developmental status rather than definitive functional diversity. However, Lmo4 is expressed in some but not all Pea3-positive neurons (that are all Lim1/Lim2-positive) at stage 35, when all motoneurons have already exited the cell cycle, arguing for a role of Lmo4 in motoneuron differentiation.

Motoneurons can also be divided into functional subsets, e.g. extensors and flexors. These two classes receive distinct sets of interneuronal inputs (Fedirchuk et al., 1999; Landmesser and O'Donovan, 1984a; O'Donovan, 1989). These distinctive patterns of inputs are already apparent at stage 25, when motor axons are still growing to their muscle targets. Furthermore, the identity is intrinsic to motoneurons; anteroposterior limb bud rotation does not change interneuronal inputs (Landmesser and O'Donovan, 1984b; Milner et al., 1998; Vogel, 1987) (for a review, see Landmesser, 2001). Interestingly, of the five motor pools we studied, Lmo4 is expressed predominantly in those supplying extensor muscles. Approximately 90% of motoneurons supplying adductor, external femorotibialis and posterior iliotibialis muscles (all extensors) express Lmo4. Similarly, the Lim3-positive LMC neurons in the brachial cord that supply the rhomboideus muscle, another extensor, are also Lmo4 positive (data not shown). By contrast, the two flexor pools we labeled, sartorius and internal femorotibialis, have lower levels of Lmo4 expression (15% and 40%, respectively). It will be interesting to be determine if Lmo4 is an intrinsic marker for motoneurons supplying extensor versus flexor muscles.

Function of Lmo4

Lmo4 can regulate the transcriptional activities of LIM homeodomain factors in several ways. Lmo transcriptional regulatory factors lack a DNA binding domain but contain two protein-protein interaction LIM domains. Lmo proteins can compete for NLI with LIM homeodomain transcription factors, and thereby regulate the formation of LIM homeodomain/NLI complexes and their transcriptional activity. A recent study has shown that *Drosophila* Lmo can bind to Chip with higher affinity than the LIM homeodomain of Apterous and thereby regulate Apterous activity levels in vivo (Weihe et al., 2001). Whether there is a differential affinity to NLI between Lmo4 and other LIM homeodomain proteins is not yet known.

Lmo4 may also compete for other co-factors besides NLI that are specific for individual LIM homeodomain proteins and could thus regulate the expression of downstream target genes. For example, by expressing chimeric LIM domains derived from different Islet family members (i.e. Isl1, Isl2 and ISL3) in zebrafish, Okamoto and his colleagues concluded that Isl2 probably forms a transcriptional complex with an Isl2-specific co-factor, in addition to NLI. Interaction with an Isl2-specific co-factor could contribute to the role of Isl2 in the differentiation of primary motoneurons, neuronal positioning, peripheral axonal outgrowth and neuronal transmitter expression in zebrafish (Segawa et al., 2001).

Combinatorial interactions of Lmo4 with other transcription factors might provide additional mechanisms for the regulation of transcription during neuronal development. In enkaphalin-producing neurons, Lmo4 interacts with the transcription factor DEAF1 (deformed epidermal autoregulatory factor 1) (Sugihara et al., 1998). DEAF1 has been implicated in opioid production by regulating enkaphalin transcription through a retinoic acid-responsive element. Interestingly, in the fetal and adult mouse brain, Lmo4 expression is region specific: high

levels of expression are present in the limbic system and in regions involved in autonomic, motor and neuroendocrine regulation (Huggenvik et al., 1998). Recently, studies in breast cancer cell lines have demonstrated that Lmo4 expression is upregulated and forms a multiprotein complex with CtIP and BRCA1 (Sum et al., 2002; Visvader et al., 2001). A role for BRCA1 in neurons has not been explored.

Different LIM homeodomain proteins are known to activate different downstream target genes (Hobert and Westphal, 2000). The pattern of neuronal generation in the ventral neural tube is achieved primarily by the spatially restricted expression of transcriptional repressors (Muhr et al., 2001). By modulating the transcriptional activity of LIM homeodomain proteins, Lmo4 is likely to be involved in the specification of motor neuronal identity. Its restricted expression in subsets of muscle sensory neurons suggests that it contributes to the specification of sensory neurons as well.

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