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Authors: Hara, Kenji, Satoh, Ken, and Ide, Hiroyuki

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Apical Ectodermal Ridge-Dependent Expression of the Chick 67 kDa Laminin Binding Protein Gene (*cLbp*) in Developing Limb Bud

Kenji Hara[1,3]*, Ken Satoh[2] and Hiroyuki Ide[1]

[1]*Biological Institute, Tohoku University, Sendai, Japan*
[2]*Research Institute of Developmental Aging and Cancer, Tohoku University, Sendai, Japan*

ABSTRACT—Apical ectodermal ridge (AER)-mesoderm interaction is important for morphogenesis in the developing chick limb bud. Genes whose expression is dependent upon the presence of AER, are likely to play important roles in the AER-mesoderm interaction. We report here the gene expression pattern of the chick homolog of the 67 kDa laminin binding protein (LBP), which is a non-integrin laminin receptor whose function relates to cell attachment, spreading, and polarization. Northern analysis showed that a single 1.4 kb transcript exists in stage 20 limb buds and which is dramatically reduced 24 hr after removal of AER. *In situ* hybridization analysis revealed that the chick 67 kDa laminin binding protein gene (*cLbp*) was expressed in the mesodermal region overlapping the *Msx1*-expressing domain and in the AER in early stage limb buds. Expression in the mesoderm was gradually restricted to the distal region underneath the AER as development proceeds. The expression in the limb mesoderm could be induced by local application of FGF-2 which could thus mimic the AER functions. These results indicated that the expression of *cLbp* depends on AER signals and that the 67 kDa non-integrin receptor binding to laminin plays a role in the AER-mesoderm interaction.

INTRODUCTION

During the development of the chick limb bud, a reciprocal interaction takes place between the apical ectodermal ridge (AER) and the underlying mesoderm. The AER is responsible for inducing wing outgrowth and maintaining the underlying mesoderm in a labile, undifferentiated state. These undifferentiated and proliferating cells underlying the AER compose a region referred to as the "progress zone" which is the site of positional fate assignment in the limb (Summerbell *et al.*, 1973). The limb bud mesoderm, on the other hand, maintains both the specialized morphology and functional properties of the AER (Hinchliffe and Johnson, 1980; Fallon *et al.*, 1983). Although the morphogenic importance of the AER-mesodermal interaction is now well established, the underlying mechanisms are not yet fully understood. Growth factors, homeobox-gene products, and the extracellular matrix (ECM) are all strongly implicated in the signaling process of the AER-mesodermal interaction (see for example Tomasek and Brier, 1986; Muneoka and Sassoon, 1992).

A number of growth factors, including FGF-2, which is a member of the fibroblast growth factor (FGF), are expressed in the AER and the distal mesoderm of the developing limb bud (Ralphs *et al.*, 1990; Niswander *et al.*, 1993; Savage *et al.*, 1993; Crossley *et al.*, 1996). It has been shown that FGF-2 can mimic the growth stimulating effects of the AER on progress zone cells (Riley *et al.*, 1993; Fallon *et al.*, 1994). These studies suggest that FGF-2 could substitute for some AER functions.

The *msh*-like homeobox-containing gene, *Msx1*, is normally expressed in the AER and the progress zone in early stage limb buds (Yokouchi *et al.*, 1991). Expression in the progress zone is controlled by signals emanating from the AER (Ros *et al.*, 1992), and mesodermal expression of *Msx1* can be maintained by FGF-2 (Watanabe and Ide, 1993). *Msx1* has therefore been considered to be involved in AER-mesodermal interactions by maintaining progress zone cells in an uncommitted state (Robert *et al.*, 1991; Ros *et al.*, 1992).

The role of the ECM in morphogenetic tissue interactions has been studied extensively. ECM components regulate many aspects of cell behavior including motility, morphology and gene expression (Adams and Watt, 1993). In addition, the ECM can regulate the expression and activity of certain growth factors, including members of the FGF family (Yamaguchi *et al.*, 1990; Streuli *et al.*, 1993; Mason, 1994). Therefore matrix molecules may act both directly and indirectly to regulate cell behavior during development. Laminin, a major component

* Corresponding author: Tel. +81-75-751-3993;
FAX. +81-75-751-3992.
[3] Present address: The Institute for Virus Research, Kyoto University, Kyoto 606–01, Japan.

of the basement membrane and extracellular matrix, functions in cell adhesion, proliferation, and differentiation (Timpl, 1989; Mecham, 1991). The glycoprotein laminin is involved in cancer metastases, as well as tumor invasiveness (Terranova *et al.*, 1983; Wewer *et al.*, 1986), and malignant cells often display aberrations in this protein (Kanemoto *et al.*, 1990; Yamamura *et al.*, 1993). It is also clear that laminin plays crucial roles in lung morphogenesis (Schuger *et al.*, 1990). In chick limb bud laminin is expressed in the subectodermal basement membrane, especially at the base of the AER (Critchlow and Hinchliffe, 1994), indicating a possible role of laminin in AER-mesodermal interaction.

Originally isolated from extracts of mammalian cells the 67 kDa laminin binding protein (67 kDa LBP) has been studied as a prototypic non-integrin ECM receptor (Wewer *et al.*, 1986). This protein binds the peptide sequence YIGSR, found in the β1 chain of laminin, with higher affinity than the integrins (Graf *et al.*, 1987; Bushkin-Harav *et al.*, 1995; Landowski *et al.*, 1995a). It has also been demonstrated that the expression of the 67 kDa protein and its mRNA is down-regulated by the differentiation of human colon carcinoma cells (Yow *et al.*, 1988; Mafune *et al.*, 1990) and human neuroblastoma cells (Bushkin-Harav *et al.*, 1995), and that hence its interaction with laminin might play a role in the attachment and spreading of carcinoma cells (Cixe *et al.*, 1991; Hand *et al.*, 1985; Wewer *et al.*, 1987) and the polarization of MDCK cells (Salas *et al.*, 1992).

In order to study genes with important functions in AER-mesodermal interaction, an AER-free limb bud cDNA library (AER(–)) subtracted from a stage 20 wing bud cDNA library (AER(+)) was screened. Sequencing of nine cDNA clones, which were specific to or enriched in AER(+) library, revealed three to be mitochondrial genes, two genes encoding respiratory enzymes, one ribosomal gene, and two genes which showed no significant homology to known proteins. The ninth clone was a partial cDNA of the 67 kDa laminin binding protein gene.

The purposes of the present work are to describe the expression pattern of the chick LBP gene (*cLbp*) in the limb bud, and to analyze the regulation of its expression by the AER and FGF-2 in an attempt to extend our knowledge about the significance of cLBP in AER-mesodermal interaction.

MATERIAL AND METHODS

Subtractive PCR

The subtractive PCR was carried out as described by Nakayama *et al.* (1996). RNA was extracted from wing buds at stage 20 (AER(+)) and wing buds 24 hr after AER removal (AER(–)), by the acid guanidinium thiocyanate-phenol-chloroform (AGPC) method (Chomczynski and Sacchi, 1987). Poly(A)$^+$ RNA was purified from each sample on oligotex-dT 30 super (Takara), and double-stranded cDNAs were synthesized using a cDNA synthesis kit (Pharmacia). cDNAs were the digested with restriction enzyme *Rsa*I (Takara) to produce fragments for subtraction. The digested DNA fragments of AER(+) and the AER(–) were ligated respectively with M13-forward (gtaaaacgacggccagtgag) and M13-reverse (cggaaacagctatgaccatg) adapters. An AER(+) specific library was constructed by subtraction of AER(–) from AER(+), then amplifying by PCR using M13-reverse primer to amplify specifically subtracted AER(+). The amplified fragments were subcloned into pCR-TMII plasmid vector (Invitrogen). Se-

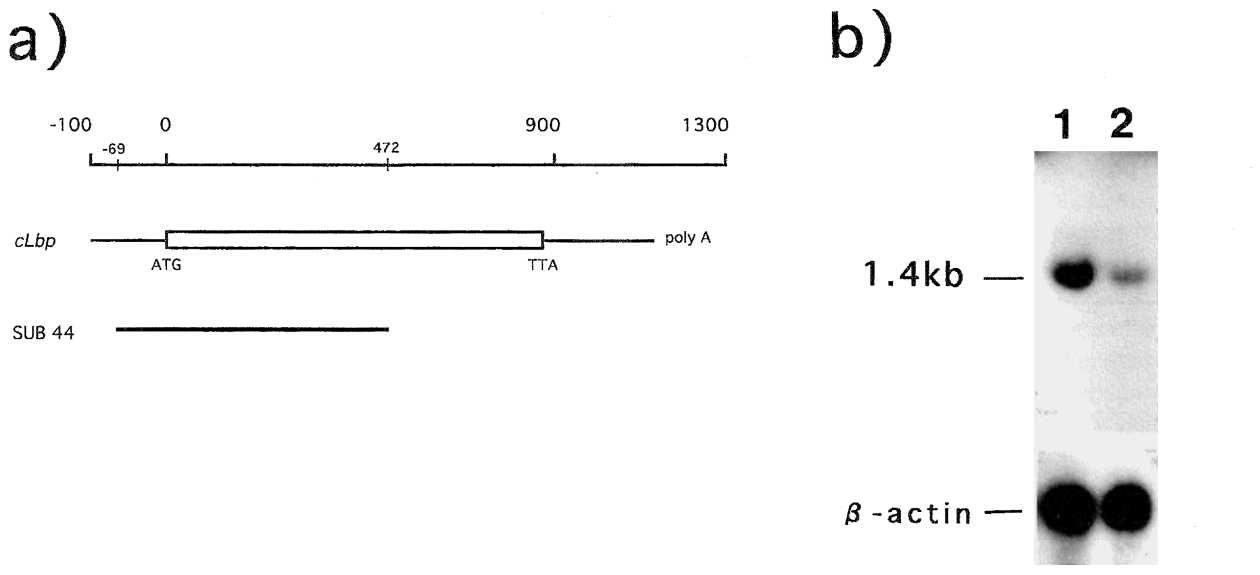

Fig. 1. (**a**) Schematic diagram of chick 67 kDa laminin binding protein cDNA (*cLbp*). The full length cDNA map is drawn according to GenBank X94368. The boxed region indicates the translated portion. Clone SUB44 was obtained by subtractive PCR, which overlapped the sequence from –69 to 472 of *cLbp*. (**b**) Northern blot analysis. poly(A)$^+$ RNA (10 μg/lane) from stage 20 limb buds (AER(+)) (lane 1) and the wing buds 24 hr after AER removal at stage 20 (AER(–)) (lane 2) were loaded. The blot was hybridized with the SUB44 probe after final washes of the filters in 0.1 × SSC, 0.1% SDS at 45°C. The probe detects a single band of about 1.4 kb. The blot was rehybridized with a chicken β-actin probe to control for RNA content in each lane.

quences of the clones were determined by the dideoxy chain-termination method using a sequencing kit (Amersham) and auto sequencer (HITACHI).

Databases were searched with the BLAST program (Altschul *et al.*, 1990) using the NCBI network service.

FGF-2 application in the chick wing bud mesoderm

Stage 20 wing buds were dissected and placed in 1% trypsin for 30 min. at 4°C to remove the ectoderm (Aono and Ide, 1988). The denuded mesoderm fragments were kept in F12 medium (Nissui) containing 1% FCS at 37°C.

For FGF-2 application, Affi-Gel beads (200-250 μm diameter; Bio-Rad) were soaked in 2 μl of 0.1 μg/ml FGF-2 (R&D systems) for at least 1 hr at room temperature before application. A small slit was made in the denuded mesoderm with a needle and a bead was inserted into it. The operated mesoderm fragments were incubated in the F12 medium containing 1% FCS under conditions as reported previously (Aono and Ide, 1988) for 24 hr, fixed in 4% paraformaldehyde and then examined for gene expression.

***In situ* hybridization**

In situ hybridization was performed using digoxigenin-labeled probes following the procedures of Yokouchi *et al.* (1991), and whole-mount *in situ* hybridizations were carried out as described by Yonei *et al.* (1995).

RESULTS

The SUB44 cDNA clone was obtained by subtraction of the AER(–) cDNA from AER(+) cDNA library. By sequence analysis the clone was identified as fragment of the chick laminin binding protein gene (*cLbp*) (Fig. 1a). Northern blot hybridization analysis revealed that the cLBP mRNA was enriched but not specific in AER(+) (Fig. 1b).

Expression pattern of *cLbp* in developing embryo

The spatial expression pattern of *cLbp* was determined by *in situ* hybridization. We hybridized adjacent sections with the prospective sense and anti-sense probes for *cLbp* fragment, SUB44 (Fig. 1a). Control embryos hybridized with a sense probe did not show signals (not shown).

Remarkable expression of *cLbp* in stage 24 embryos could be observed in branchial arches (maxillary, mandibular, and hyoid arch), dermomyotome, and in the distal margin of limb buds (Fig. 2a). In sections through the otocysts, *cLbp* was expressed in the mesoderm of the hyoid arch, being especially strong in areas underneath ectoderm (Fig. 2b). Weak

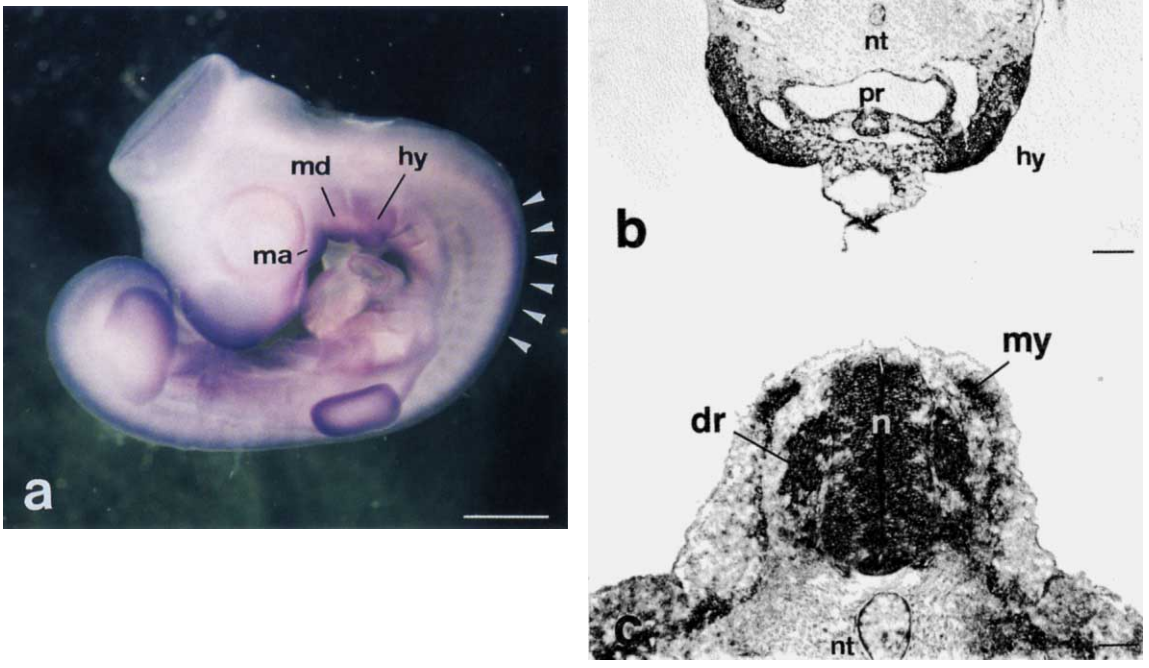

Fig. 2. Analysis of *cLbp* expression in stage 24 chick embryo. (**a**) Whole mount views. Arrowheads indicate myotome. (**b**) Transverse section at the hindbrain level, and (**c**) at the trunk level. dr, dorsal root ganglia; hb, hindbrain; hy, hyoid arch; ma, maxillary; md, mandibular; my, myotome; n, neural tube; nt, notochord; ot, otocyst; pr, pharynx. Bars= 1mm for (**a**); 100 μm for (**b**) and (**c**).

expression was also observed in the neural tube and the otocyst. In sections through the trunk, *cLbp* was strongly expressed in the neural tube, dorsal root ganglia, and nerve roots (Fig. 2c). Neural crest and notochord was negative. The dorsal lateral region of the somite was positive, though the sclerotome was negative at this stage (Fig. 2c). The precursors of the limb musculature (arrowheads in Fig. 3c), which emigrated from the dermomyotome into the limb bud (Chevallier *et al.*, 1977; Williams and Ordahl, 1994), were positive. Mesonephrons also expressed *cLbp* strongly.

The expression patterns of *cLbp* in various tissues of stage 16-29 embryos are summarized in Table 1. The expression in dermatome decreased by stage 25. The differentiating sclerotome was positive until stage 18 (cf. Fig. 3a, b). The other tissues showed the same expression pattern throughout stages 16 to 29.

Expression pattern of *cLbp* in the developing limb bud

The prospective wing mesoderm of stage 16 embryos did not express cLBP mRNA (not shown). The expression was first detected at stage 18 in the whole mesoderm of the limb bud (Fig. 3a), but not in the flank region (Fig. 3b).The prospective AER was positive (arrow in Fig. 3a), but non-ridge ectoderm was negative. In stage 20 limb buds, cLBP mRNA was transcribed broadly in the mesoderm of the distal region, being most abundant in the cells underneath the dorsal ectoderm (Fig. 3c). The AER, especially cells at the epidermal-mesodermal interface, was positive (arrow in Fig. 3c), whereas non-ridge ectoderm was negative. Hybridization to a horizontal section (Fig. 3d) showed no difference in expression along the anteroposterior axis of limb bud. The expression was gradually restricted to the distal margin during limb development. At stage 24, expression remained in the region of the distal mesoderm underneath the ectoderm, but disappeared in the proximal mesoderm (Fig. 5e). In the proximal region the expression was observed the skeletal muscle, but not in the developing skeletal elements including the perichondrium (Fig. 5f). By stage 29, *cLbp* expression had decreased (Table 1).

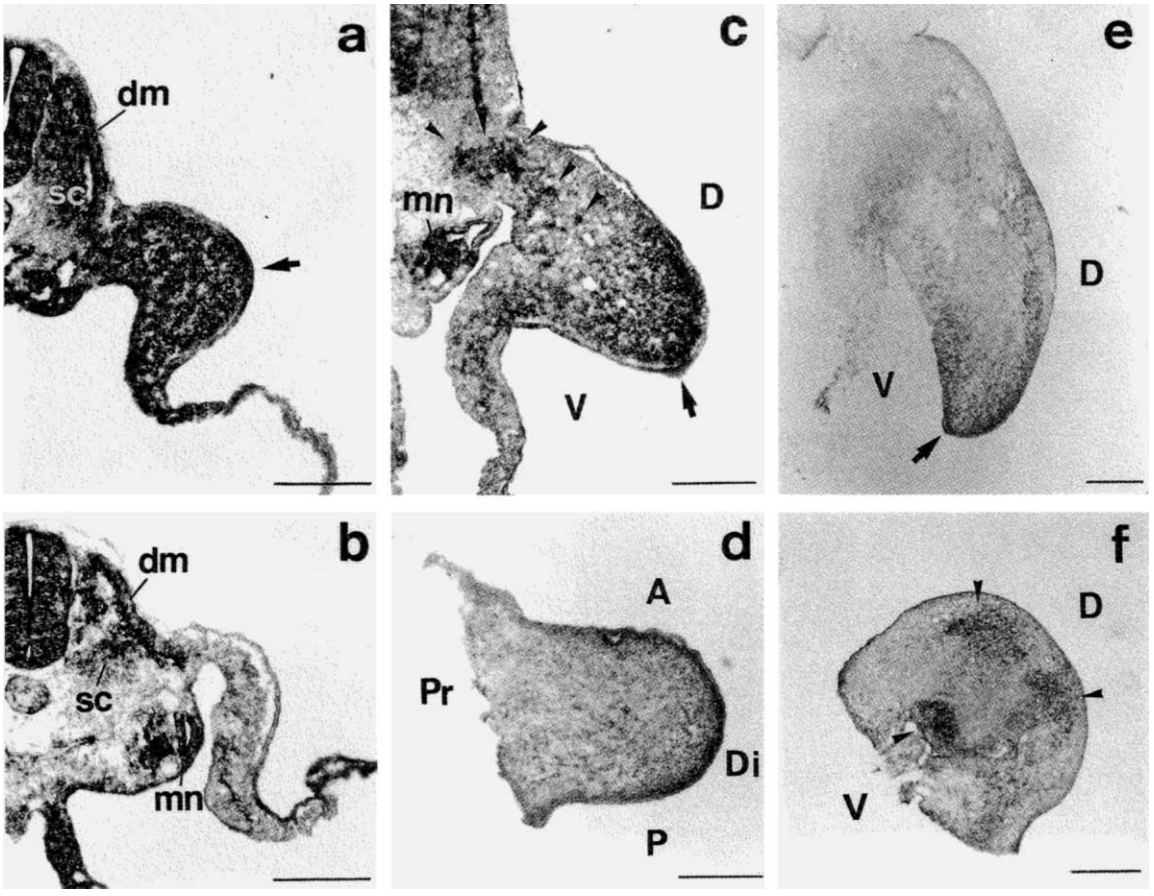

Fig. 3. *cLbp* expression in developing wing bud (see text). Adjacent section at the wing bud level (**a**), and trunk level (**b**) of a stage 18 embryo. Arrow indicates the prospective AER. dm, dermomyotome; sc, sclerotome; mn, mesonephros. (**c**) Transverse section from stage 20 embryo through the wing region. Arrow indicates AER. Arrowheads indicate the precursors of the limb musculature. mn, mesonephros. D, dorsal; V, ventral. (**d**) Horizontal section of a wing bud in stage 20 embryo. This section is at a slightly dorsal level and so does not contain the AER. A, anterior; P, posterior; Di, distal; Pr, proximal. (**e**) Transverse section of a wing bud in stage 24 embryo, Arrow indicates AER. (**f**) Parasagittal section at the proximal region of a stage 24 wing bud. Arrowheads indicate skeletal muscles. Bars = 300 μm for (**a**) and (**b**); 500 μm for (**c**)-(**f**).

Table 1. Summary of *cLbp* expression*

	Stage				
	16	18	22	25	29
Limb					
Ectoderm	–	+/d	+/d	+/d	–
Dorsal	nd	–	–	–	–
Ventral	nd	–	–	–	–
AER	nd	+	+	+	nd
Mesoderm	–	+	+/d	+/d	±
Peripheral	nd	+	+	+	±
Muscle mass	nd	nd	nd	+	+
Chondrogenic core	nd	nd	nd	–	–
Trunk					
Neural tube	+	+	+	+	+
Peripheral nerves	+	+	+	+	+
Dorsal root ganglia	+	+	+	+	+
Neural crest	–	–	–	–	–
Notochord	–	–	–	–	–
Somite					
Myotome	+	+	+	+	+
Sclerotome	+	+	–	–	–
Dermatome	+	+	+	±	±
Mesonephros	nd	+	+	+	+

*: –, not stained; ±, weekly stained; +, stained; +/d, stained distally but not proximally; nd, not determined.

AER-dependent expression of *cLbp*

The cLBP mRNA significantly decreased in the AER-free limb bud as compared with normal controls, suggesting a possibility that *cLbp* transcription could be maintained by the AER. *Msx1* is expressed in the AER and in the mesoderm underneath it (Yokouchi *et al.*, 1991), and expression is controlled by signals emanating from the AER (Ros *et al.*, 1992). Therefore, we compared *cLbp* and *Msx1* expression patterns by whole mount *in situ* hybridization.

cLbp expression was stronger in the marginal zone of the limb bud, but disappeared in the flank region (Fig. 4a). The expression pattern was similar to that of *Msx1* in early stage limb buds (Fig. 4b). At stage 25, expression was observed in areas distal to the autopodium, and also remained in the posterior marginal zone (Fig. 4c). These patterns were broader than the *Msx1* expression which was restricted to the distal margin and the interdigits (Fig. 4d). Both *cLbp* (5 cases; Fig. 4e) and *Msx1* (3 cases; Fig. 4f) expressions were dramatically reduced in the limb bud 24 hr after surgical removal of the AER at stage 20. This result supports data from Northern hybridization (cf. Fig. 1b). However, the expression of *cLbp* could still be observed 12 hr after AER removal (not shown), whereas the expression of *Msx1* was undetectable in the distal mesoderm by 6 hr after the operation (Ros *et al.*, 1992).

FGF-2 has been shown to mimic the functions of the AER (Riley *et al.*, 1993; Fallon *et al.*, 1994). To determine whether FGF-2 is able to induce *cLbp* expression in the mesoderm of stage 20 limb bud, a heparin bead soaked in FGF-2 (0.1 μg/ml) was applied to the mesodermal mass lacking an AER (7 of 10 cases). After 24 hr, expression was induced in the mesoderm surrounding the FGF-2 bead (Fig. 5a), whereas little signal was detected in control fragments (8 cases; Fig. 5b).

DISCUSSION

Characteristics and possible role of cLBP in developing limb bud

The 67 kDa laminin binding protein (67 kDa LBP) was originally isolated from the extracts of mammalian cells by affinity chromatography on laminin-Sepharose columns (Wewer *et al.*, 1986). This protein binds the peptide sequence YIGSR, found in the β1 chain of laminin, with higher affinity than the integrins (Graf *et al.*, 1987; Bushkin-Harav *et al.*, 1995; Landowski *et al.*, 1995a), and might play a role in cell attachment, spreading, and polarization (Hand *et al.*, 1985; Wewer *et al.*, 1987; Cixe *et al.*, 1991; Salas *et al.*, 1992). A partial cDNA clone for the human 67 kDa LBP was originally selected from an expression library by screening with a monoclonal antibody raised against human laminin (Wewer *et al.*, 1986). Subsequently, full-length cDNA clones were obtained, from various mammals, by investigators specifically interested in laminin binding proteins (Rao *et al.*, 1989; Grosso *et al.*, 1991), as well as groups studying gene expression in transformed cells (Yow *et al.*, 1988; Satoh *et al.*, 1992a, b; Kondoh *et al.*, 1992), translational control in mouse cells (Chitpatima *et al.*, 1988), and development of the embryonic eye (Rabacchi *et al.*,1990). All the cDNAs obtained encode proteins of estimated molecular weight between 32-34 kDa, which corresponds to the 32 kDa precursor of the 67 kDa LBP in human cells (Landowski *et al.*, 1995b), and the amino acid sequences are highly conserved. These proteins lack the signal sequences or simple hydrophobic domains that would be expected in a typical trans-membrane protein (Grosso *et al.*, 1991). cLBP had no distinct N-terminal signal peptide sequence following the putative initiation site (GenBank X94368). However, Landowski *et al.* (1995a) shows the expression of the 67 kDa LBP on the cell surface using a homotypic overexpression system. It appears to form a homodimer of 32 kDa subunits (Landowski *et al.*, 1995b), associates with membranes and interacts with elements of the cytoskeleton (Brown *et al.*, 1983; Massia *et al.*, 1993; Keppel and Schaller, 1991). These observations suggest that LBP function may be dependent on posttranslational modifications responsible for surface localization and laminin-binding characteristics (Landowski *et al.*, 1995a).

In chick limb bud, laminin is present in all regions of the subectodermal basement membrane as a clearly defined line and also in the distal mesoderm (Critchlow and Hinchliffe, 1994). We showed that *cLbp* was localized in the AER and the distal mesoderm underneath it (Fig. 3e), whereas β1 integrin, which is a subunit for laminin receptors (von der Mark *et al.*, 1991; Sonnenberg *et al.*, 1993; Thorsteinsdottir *et al.*, 1995), localized along the entire epidermal-mesodermal interface (Critchlow and Hinchliffe, 1994), suggesting that cLBP might have different roles from the integrin receptor in epidermal-mesodermal interaction. Salas *et al.* (1992) shows that the 67 kDa LBP is involved in the acquisition of apical polarity

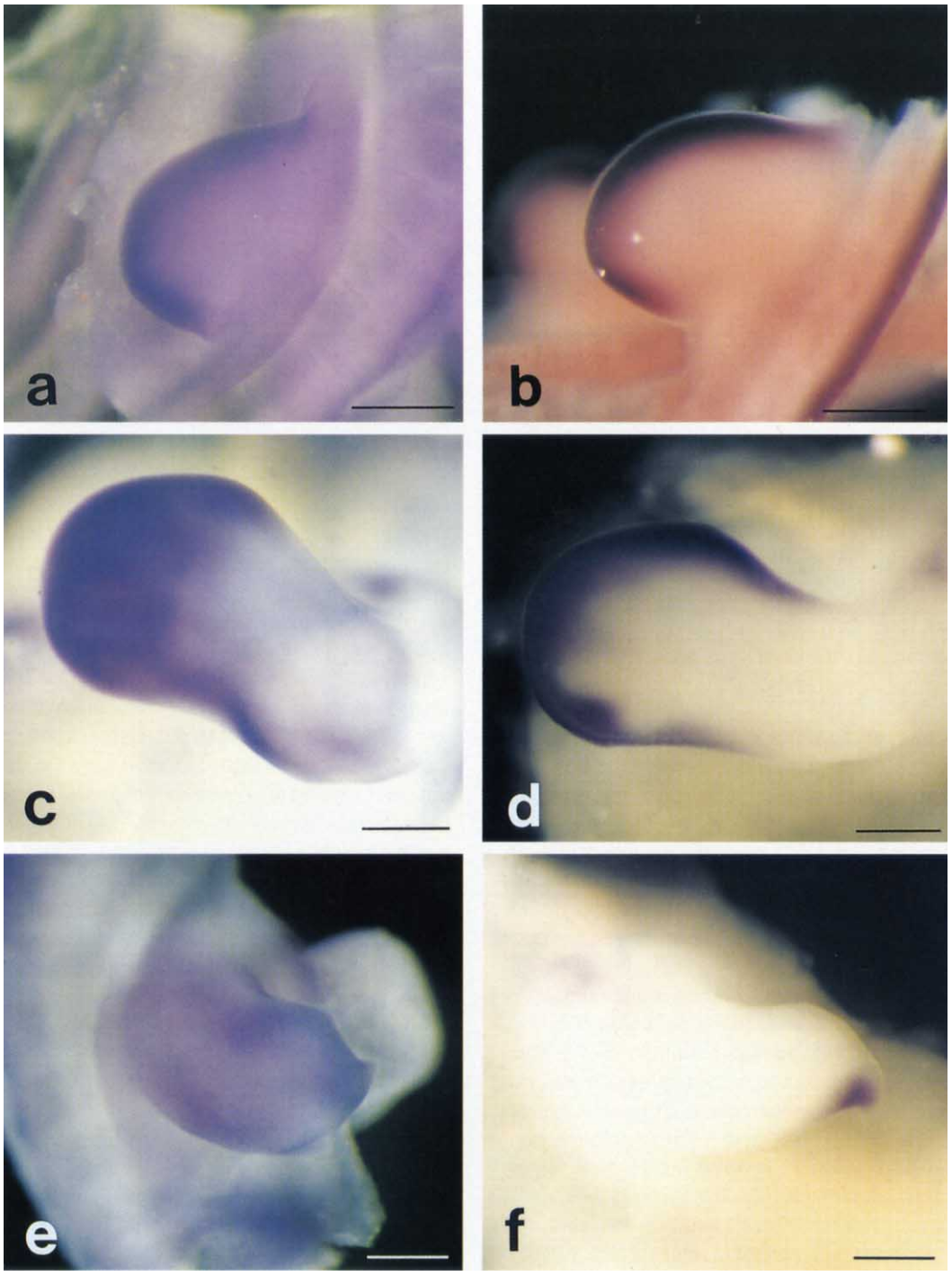

Fig. 4. Comparison of *cLbp* and *Msx1* expression in limb buds. During early stages of limb development *cLbp* is expressed in the peripheral zone, overlapping *Msx1*. Shown are whole-mount *in situ* hybridizations of stage 22 limb buds probed for *cLbp* (**a**), *Msx1* (**b**). At stage 25 *cLbp* expression (**c**) is broader than *Msx1* expression which is restricted to the distal margin and interdigit regions (**d**). Both *cLbp* (**e**) and *Msx1* (**f**) expression were dramatically reduced in the limb bud 24 hr after AER removal at stage 20. Bars = 500 μm.

of MDCK cells. These results indicate that *cLbp* might transduce an ECM-signal to the cell responsible for the organization of the apical region in the limb bud. In addition there is evidence that the ECM also plays a role in establishing morphological differences between AER and non-ridge epithelia in the limb bud (Tomasek and Brier, 1986). cLBP may therefore also participate in maintaining the special AER structure.

The expression of *cLbp* in the muscle precursors (cf. Fig. 3c) and muscle masses (cf. Fig. 3f) might be of significance in relation to the formation of the muscle masses in the limb bud. Laminin is concentrated in the muscle masses in the limb bud (Solursh and Jensen, 1988; Critchlow and Hinchliffe,

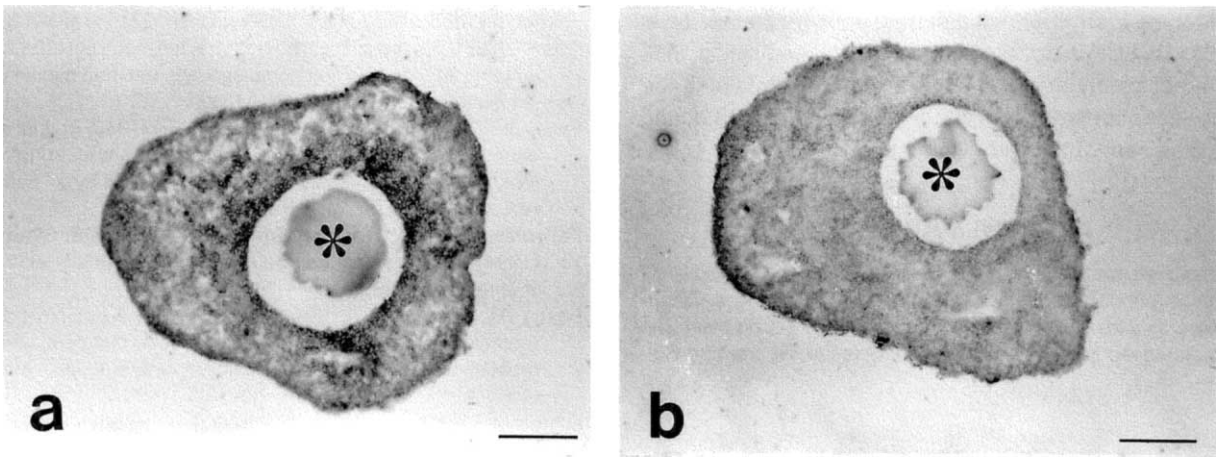

Fig. 5. Effect of FGF-2 on *cLbp* expression in chick limb mesodermal cells. (**a**) Bead (asterisk) presoaked in PBS containing FGF-2 (0.1 μg/ml) was implanted in the limb mesoderm from which ectoderm was removed in advance. (**b**) Control case with PBS-loaded bead (asterisk). Bars = 100 μm.

1994). Previous works have shown that laminin substratum could enhance myoblast adhesion, promote myoblast proliferation, and migration (Kuhl *et al.*, 1982, 1986; von der Mark and Kuhl, 1985; Ocalan *et al.*, 1988). Furthermore Foster *et al.* (1987) showed that rat skeletal myoblasts become responsive in terms of increased proliferation and differentiation to a laminin substratum at a particular stage during development. Since β1 integrin was not synthesized in the muscle masses in the wing bud (Critchlow and Hinchliffe, 1994), our data may support a role for cLBP as a functional receptor of laminin in regulating the proliferation, migration, and formation of the early muscle formation during limb morphogenesis.

On the other hand, previous studies suggested the possibility that 67 kDa LBP may have another function, serving a role in control of translation. Cytosolic protein (p40) from mouse cells, which is associated with ribosomes and polysomes, has shown similarity to the 67 kDa LBP (McCaffery *et al.*, 1990; Auth and Brawerman, 1992). Several groups have also identified proteins from rat (Tohgo *et al.*, 1994), sea urchin (Rosenthal and Wordeman, 1995), flies (Melnick *et al.*, 1993), and yeast (Davis *et al.*, 1992; Ellis *et al.*, 1994; Demianova *et al.*, 1996) with extensive sequence similarity to the 37/67 kDa LBP, and have shown that these proteins are apparently components of the ribosomal translational machinery.

Relation of *cLbp* expression to mesodermal cell differentiation in chick limb bud

Chick limb development depends on the continuous presence of the AER (Saunders, 1948; Summerbell, 1974a, b). Reciprocal interactions with the AER promote the growth of the underlying mesoderm (progress zone) and maintain it in an undifferentiated state (Globus and Vethamany-Globus, 1976; Solursh *et al.*, 1981). FGF-2 is present at high concentrations during the early stage of chick limb bud development (Munaim *et al.*, 1988; Seed *et al.*, 1988; Savage *et al.*, 1993), and it has been shown to mimic the effects of AER (Riley *et al.*, 1993; Fallon *et al.*, 1994). *Msx1* is normally expressed in the AER and the progress zone mesoderm in early stage wing buds (Hill *et al.*, 1989; Robert *et al.*, 1989; Davidson *et al.*, 1991; Yokouchi *et al.*, 1991). The expression in the limb mesoderm is controlled by signals emanating from the AER (Ros *et al.*, 1992; Robert *et al.*, 1991), and FGF-2 has been shown to maintain *Msx1* expression (Watanabe and Ide, 1993; Wang and Sassoon, 1995). Though the role of this protein itself in developing limb buds is at present unclear, myogenic cell lines that constitutively express *Msx1* have been shown to become differentiation-defective (Song *et al.*, 1992; Woloshin *et al.*, 1995). These observations are consistent with the hypothesis that the AER is involved in the maintenance of the underlying mesoderm in an undifferentiated state via the regulation of *Msx1* gene expression.

We showed that excision of the AER reduced the *cLbp* transcription level and that FGF-2 could recover the expression in mesodermal cells in a similar manner as *Msx1* (Ros *et al.*, 1992), indicating that *cLbp* expression in the limb mesoderm could be maintained by FGFs emanating from the AER. However *cLbp* expression remained in the distal mesoderm 12 hr after excision of the AER (not shown), although by this point *Msx1* was undetectable (Ros *et al.*, 1992). The expression patterns of *cLbp* and *Msx1* differ during normal development: expression of *Lbp* is broader than that of *Msx1*. These findings suggest that *cLbp* expression would not be regulated through the *Msx1* cascade. *cLbp* expression in the chick limb bud was distributed as a gradient with the highest levels distally, and almost overlapped the *Msx1* expression pattern. cLBP transcripts are also found in undifferentiated mesoderm at the tips of the facial primordia in a similar fashion to *Msx1* (Brown *et al.*, 1993). It has been demonstrated that the increased amount of 67 kDa LBP upregulates properties of malignant cells such as high metastatic potential and rapid growth (Cixe *et al.*, 1991; Hand *et al.*, 1985; Wewer *et al.*, 1987), and that the expression of the 67 kDa LBP and its mRNA are dramatically reduced in differentiated neuroblastoma cells (Bushkin-Harav *et al.*, 1995) and colon carcinoma (Yow *et al.*,

1988; Mafune *et al.*, 1990). It therefore appears that the level of the 67 kDa LBP is closely related to cell differentiation, indicating a possibility that cLBP has a role in maintaining the progress zone mesoderm in an undifferentiated state during the development of the chick limb bud.

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