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Original Article

Isolation and characterization of a *Xenopus* gene *(XMLP)* encoding a MARCKS-like protein

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ABSTRACT We have identified a cDNA coding for a *Xenopus* MARCKS-like protein (XMLP) from a cDNA library prepared from activin-treated ectoderm. Using whole-mount *in situ* hybridization and RT-PCR, we found *XMLP* maternal transcripts during the cleavage stages. After MBT, the signals were restricted to the neural plate. Subsequently *XMLP* was expressed predominantly in the brain, somites and pronephros. Ectopic expression of *XMLP* resulted in eye and axis defects and in a change of the expression pattern of *Krox 20*, a neural marker for rhombomeres 3 and 5. Injected *XMLP* caused apoptosis. It was characterized by loss of intercellular adhesion contacts, transient plasma membrane ruffling at gastrula, and epithelial disruption at tailbud stage. Overexpression of mutant *XMLPs* showed that this phenotype was correlated with its putative PSD domain and glycine at position 2. The embryos injected with a morpholino oligo complementary to *XMLP* mRNA showed malformations of the anterior axis and eye defects. Extirpation experiments indicated that the phenotypes might be correlated with disturbed morphorgenetic movements rather than an inhibition of induction process. Overexpression of *XCYP26* resulted in a shift of the expression pattern of *XMLP*. In the early tailbud stage (stage 20) the signal stripe in the *XCYP26* injected half of the embryo got diffuse or even disappeared. This observation suggests that retinoic acid plays an important role in the regulation of *XMLP*. Our results suggest that XMLP might participate in pattern formation of the embryonic axis and the central nervous system.

KEY WORDS: *XMLP, PKC, XCYP26, Krox20, apoptosis, morpholino*

Introduction

Phosphorylation of intracellular substrates by protein kinase C (PKC), which composes a family of diacylaglycerol-activated and Ca^{2+}-dependent serine / threonine related protein kinases, is an impetus for a wide range of cellular processes including differentiation, mitogenesis, neurotransmission, and hormone secretion. Up to now at least 10 subtypes of this family have been found and each subtype has shown different enzymological properties and distinct cell type distribution (for review see Nishizuka, 1984, 1995). In *Xenopus*, it has also been demonstrated that PKC is involved in the response to endogenous inducing signals during neural induction (David *et al.*, 1987; Otte *et al.*, 1988; for review see Grunz, 1999a). One of the most prominent intracellular substrates for PKC is the myristoylated alanine rich C kinase substrate (MARCKS), which can be phosphorylated in many cell types by PKC. The members of MARCKS share three conserved domains, a myristoylated consensus following the glycine residue at position 2 in the amino terminus, the site of intron splicing, and the phosphorylation site domain (PSD), also called effector domain (ED) which contains three of four serines that are the only residues known to be phosphorylated by PKC in this domain. MARCKS related proteins have also been identified in mouse, rabbit and human, sharing striking similarity with MARCKS. They were termed as MARCKS like protein (MLP), also known as F52 or MacMARCKS. MARCKS is a ubiquitous 32-kDa protein, whereas *MLP* is mainly expressed in brain and reproductive tissues (Aderem, 1992; Blackshear, 1993).

MARCKS behaves as a PKC substrate and binds to calmodulin in a Ca^{2+}-dependant manner. However, there is no direct evidence showing that the protein contains a Ca^{2+} binding domain. Additionally it was demonstrated that dephosphorylated MARCKS could bind to and cross-link filamentous actin *in vitro*. Both

Abbreviations used in this paper: dpc, days post conception; ED, effector domain; MARCKS, myristoylated alanine-rich C kinase substrate; MBT midblastula transition; MLP, MARCKS like protein; MO, morpholino; ODC, ornithine decarboxylase; PKC, protein kinase C; PSD, phosphorylation site domain; RA, Retinoic acid; XCYP 26, *Xenopus* cytochrome P450 26 (RA hydroxylase); XMLP, *Xenopus* MARCKS like protein.

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1536 aaaaga<b>aataaa</b>ttttaacccccttaaaaaaaaaaaaaaaaaa
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Fig. 1. XMLP nucleotide and the deduced amino acid sequence. *The nucleotide sequence in bold in the 3'UTR is the putative polyadenylation signal. The numbers on the left indicate the positions of the nucleotides and on the right the positions of the amino acids. The stop codon is marked by an asterisk. A purine at position -3 is in agreement with the Kozak sequence (Kozak, 1986). Glycine is found at position 2. The conserved region at 22 to 27 surrounding the site of intron splicing is also found in other members of the MARCKS familiy. The domain from K78 till K99 is the putative PSD domain. The three conserved sites mentioned above are underlined.*

functions are correlated with the PSD domain (for review see Blackshear, 1993).

It has been reported that the expression of MARCKS increased sharply when Swiss 3T3 cells escaped from cell cycle and entered G0 (Herget *et al.,* 1993). Reduced expression of MARCKS has been described in various cell lines after oncogenic or chemical transformation. On the other hand, overexpression of the MARCKS in human tumor-derived choroidal melanoma cells (OCM-1) can cause down-regulation of cell proliferation with high percentage of cells arrested in G0-G1 phase (Manenti *et al.*, 1998). However the effect of MARCKS on the cellular functions is not yet well understood and even less for MLP. It is assumed that MLP has a similar function as MARCKS because both are sharing high sequence homology. Recent gene knockout studies have indicated that, at least in mice, MARCKS is essential for the normal development of the central nervous system and postnatal survival (Stumpo *et al.*, 1995). MARCKS gene knockout mice exhibited lethal neural defects during development such as defect of cerebral hemisphere fusion, disturbance of forebrain commissures formation as well as cortical and retinal lamination abnormalities of the cortex and retina. But by expression of non-myristoylated human MARCKS in this null MARCKS population all the neuroanatomical abnormalities could be rescued (Swierczynski *et al.*, 1996). Expression of a transgene containing non-myristoylation and pseudo-phosphorylation sites was able to compensate almost the entire cerebral anatomical abnormalities of the knockout mice, however these mice also exhibited profound retinal ectopia (Kim *et al.*, 1998). Chen *et al.* (1996) found that *MLP* deletion in mice prevents the closure of cranial neural tube in the developing brain characterized by embryonic exencephaly and postnatal anencephaly. It was shown in another *MLP* knockout mice system (Wu *et al.*, 1996) that the neural tube defects caused exencephaly and spina bifida. In this study, we present the characterization of a novel homologue of the MARCKS like protein (MLP) in *Xenopus* identified from an activin-treated ectoderm cDNA library. We found *XMLP* was differentially expressed during early *Xenopus* development. Ectopic expression of *XMLP* led to eye, axis defects and apoptosis. Embryos injected with antisense morpholino showed reduction of the anterior axis and eye defects. We also found a change in the expression pattern of *Krox 20* in overexpression, which might be correlated with disturbed morphogenetic movements rather than an inhibition of induction processes. Overexpression of *XCYP26* resulted in a special shift of the expression pattern of *XMLP* indicating that retinoic acid (RA) may play an important role for its regulation.

Results

Isolation of a gene encoding a Xenopus *MARCKS-like protein (XMLP)*

Large scale screening of a cDNA library prepared from activin-treated ectoderm led to identification of new *Xenopus* sequences. One of them composed of 187 predicted amino acids was related to MARCKS like proteins (MLP), which are members of MARCKS family and have been characterized in mouse, rat, human and rabbit. However, the protein sequence shows relatively low identity compared to the other three species. The predicted protein sequence contained three conserved domains, which have been found in other members of MARCKS and MLP families: A myristoylated consensus following the glycine residue at position 2 in the amino terminus, the site of intron splicing and the putative phosphorylation site domain (PSD). It was termed as *Xenopus* MARCKS like protein (*XMLP*) (Fig. 1). The full sequence was submitted to DDBJ/EMBL/Genebank with the accession number: AF187864. At the 5' end, 95 untranslated nucleotides precede the translation initiation site (Kozak, 1986) and at the 3' end the coding region ends with an in-frame stop codon at the position 658, which is followed by a 921-base untranslated sequence. A consensus

polyadenylation signal AATAAA is present upstream from the putative poly (A) tail.

The theoretical isoelectric point (pI) of XMLP is 4.38, and pI at deduced PSD is 12.06 (http://www.expasy.ch/tools/pi_tool.html), which are similar with the common character of this protein family (Blackshear, 1993, Ramsden, 2000).

Spatial and temporal expression of XMLP

Whole-mount *in situ* hybridization showed that abundant *XMLP* maternal transcripts were found during the cleavage stages in the animal half of the embryo (Fig. 2 A,B). Signals were detected until gastrula in almost all regions except of the yolk plug (Fig. 2C). Sections showed that the *XMLP* was predominantly expressed in ectoderm and mesoderm at stage 11.5 (Fig. 2D). From the late gastrula the transcripts decreased and were restricted to the neural plate (Fig. 2 E,F). Transversal sections of neurulae (stage 15) showed the presence of signals in the neuroectoderm (Fig. 2G). Subsequently, the signals have been located in the neural folds and brain area at the early tailbud stage (Fig. 2 H,I,M). Sagittal and transversal sections of this stage are shown in Fig. 2 J, K and L. At stage 34 the signals appeared in the head area, somites, gills, pronephros and renal tube (Fig. 2 O,P,Q).

RT-PCR showed that transcripts could be detected in all stages. The transcription level decreased from uncleaved egg and cleavage stages till stage 23, while it again increased slightly in the following stages and finally maintained a stable level (Fig. 3). RT-PCR analysis of adult tissues showed that *XMLP* is abundant in tested tissues. A relatively low expression level was found in intestine and kidney (Fig. 4).

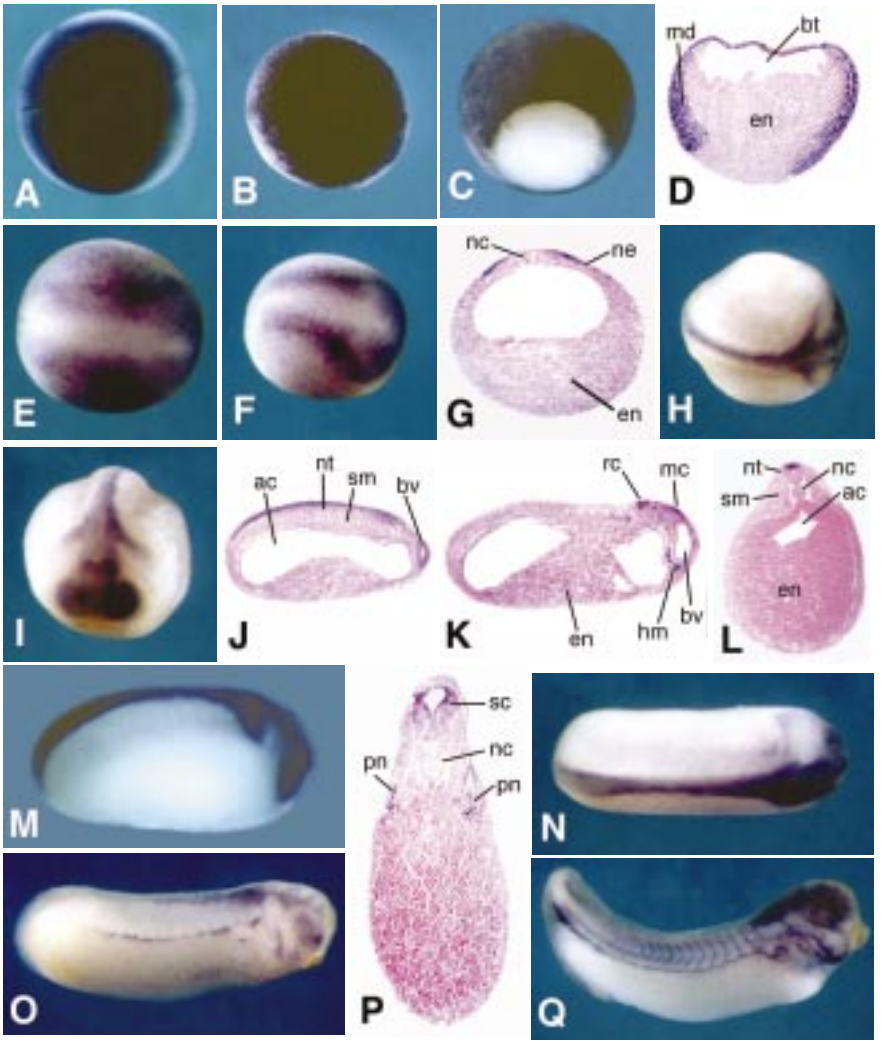

Fig. 2. Whole-mount *in situ* hybridization using XMLP antisense RNA. *Maternally expressed XMLP was present in cleave stages* **(A,B)**. *Afterwards signals were found in the ectoderm and mesoderm of gastrula stages in* **(C,D)**. *A sagittal section of stage11.5 is shown in (D). However, at neurula stage, signals were no longer evenly distributed and were restricted to neural ectoderm only* **(E,F,G)**. *Subsequently, XMLP transcripts could be found in the neural folds at early tailbud stage and presumptive brain area only* **(H,I,M)**. *The sagital section of (I) is shown in* **(J)** *and* **(K)**, *while* **(L)** *shows a transversal section of (I). In later stages XMLP was expressed in ectoderm derivatives and pronephros during stage 27 to 34,* **(N,O,Q)**. **(P)** *shows a transversal section of stage 34 (ac, archenteron; bt, blastocoel; bv, brain ventricle; ed, ectoderm; en, endoderm; hm, head mesenchyme; mc, mesencephalon; md, mesoderm; nc, notochord; ne, neural ectoderm; nt, neural tube; pn, pronephros; rc, rhombencephalon; sc, spinal chord).*

Phenotypic effects of XMLP overexpression

XMLP capped mRNA was injected in one dorsal blastomere of the 4-cell stage embryos. Checking injected gastrula, we could find some bulged cells distributed around the former injection site (Fig. 5A). Sections showed that these cells contained large nuclei (Fig. 5B). Such embryos developed quite normally, i.e., both dorsal blastopore and yolk plug can be formed correctly. This phenotype is quite similar to the phenotype of apoptosis in overexpression experiments published elsewhere (Grammer *et al.*, 2000). The typical apoptosis is the epithelia disruption and the extrusion of dead cells in the vitelline space. We also found this effect in our overexpression experiments when injected *XMLP* mRNA was higher than 0.4 ng. Bulged cells and mottled surface can be found around the injection area. Some dead cells were found between the vitelline memberane and embryo. The embryos can resilient if the apoptosis was not severe. Inspection of *Xenopus* tadpoles injected with *XMLP* RNA into one of the dorsal blastomeres of 4-cell stage embryos revealed two main morphological defects. One phenotype was a reduced lens size and abnormal shape (Fig. 5 C,D,E). Some larvae even showed cyclopia (Fig. 5F). Sections indicated the reduction of neural retina and a part of the diencephalon. In contrast to the normal embryos (Fig. 5G), there was less mesenchyme around the notochord in the forebrain area (Fig. 5H) and smaller or missing eyes (Fig. 5 H,I). Another phenotype was a bent body axis. Injection of high concentrations of *XMLP* RNA caused two phenotypes (bent axis and lens defect), which could occur simultaneously. The phenotypes are dose-dependent. 44 % (56 of 128) of injected embryos showed lens defects, 1% (1 of 128) exhibited bent axis, and 20% (25 of 128) occurred two phenotypes simultaneously when 0.2 ng *XMLP* RNA was injected into one dorsal

blastomere of 4-cell stage embryos; 27% (59 of 219) displayed lens defects, 1% (3 of 219) showed bent axis defects and 33% (71 of 219) showed lens defects and bent axis defect concurrently when the injection dose was doubled. After injection of 0.6 ng *XMLP* RNA, 23% (28 of 123) showed lens defects, 2% (2 of 123) exhibited bent axis defects, 70% (86 of 123) revealed both phenotypes concurrently. Control embryos injected with the same quantity of *LacZ* RNA developed normally.

Ectopic expression of XMLP will not change the normal autonomous differentiation of isolated dorsal blastopore lip

It is known that the isolated dorsal blastopore lip including parts of dorsal ectoderm from stage 10 embryos can autonomously differentiate into notochord, brain structures, and rudimentary eyes (Grunz, 1992, 1999b). This depends on multiple steps including cell interaction, induction and secondary cell interaction. The cooperation of genes expressed in the zone of the Spemann organizer including *goosecoid, chordin, Xnr3,* and *noggin* drives ectoderm to differentiate into dorsal structures and the central nervous system (for review see Grunz, 1997). To investigate if lens

Fig. 3. Temporal expression pattern of XMLP at different stages analyzed by RT-PCR. *Transcripts could be detected at all stages (stage 1-40, Nieuwkoop and Faber, 1975) especially at a high level in stage 8, when zygotic gene expression just starts. The analysis also revealed a decrease of the transcription level from uncleaved egg to stage 23, while it increased slightly in later stages.*

defects in whole embryos are the result of specific inhibition after overexpression of *XMLP*, we performed the following experiments. Explants consisting of dorsal blastopore lip and adjacent dorsal ectoderm were isolated from injected embryos at stage 10, and cultured in Holtfreter solution until control embryos reached stage 40. Sections showed that these explants had differentiated into notochord, brain, and eye structure similar to isolated dorsal blastopore lips from uninjected controls (Fig. 5 J,K). The results suggest that *XMLP* does not trigger a specific inhibition of the eye anlage (Fig. 5K). Therefore the results support the view that the inhibition of eye structures in injected whole embryos is the result of disturbed morphogenetic movements rather than a specific inhibition of eye formation.

A morpholino oligo (MO) against XMLP is effective when injected into cleaving embryos

Recently, a new type of antisense oligos called morpholino was used in loss-of-function studies of β-catenin (Heasman *et al.*, *2000*). It provides the following advantages over phosphorothioates, excellent sequence specificity, reliable activity inside the cell, complete resistance to nucleases (Summerton *et al.,* 1997). We studied loss-of-function of *XMLP* by injecting MO XMLP into two-cell stage embryos or two dorsal blastomeres of four-cell stage embryos. Most of injected embryos showed eye defect, shorten anterior axis and a mild bent axis (Fig. 6A and Table 1). Up to 24 ng MO XMLP was introduced into embryos in order to get significant results. No significant toxic effects were observed after injection of mopholino negative control.

β-catenin MO was employed as positive control by being injected either into two blastomeres at the 2-cell stage or two dorsal animal blastomeres of 8-cell stage embryos. Embryos injected in two dorsal animal blastomeres with 4 ng ß-catenin MO at 8-cell stage showed a reduction of the head area and anterior axis (Fig. 6E). Injection of 8 ng MO ß-catenin into each blastomere of two-cell stage embryos prevented any axis formation (not shown). Since MO XMLP antisense oligo binds the *XMLP* mRNA with high specificity, MO XMLP should strictly compensate the phenotype induced by *XMLP* mRNA. To test the specificity of the MO XMLP binding to *XMLP* mRNA, synthetic 0.8 ng *XMLP* mRNA together with 16 ng MO XMLP was co-injected to the two dorsal blastomeres of 4-cell stage embryos. The mRNA did not contain the portion of the 5' UTR of *XMLP* so that MO XMLP could not bind the foreign *XMLP* mRNA. Inspecting the injected embryos at neurula stage, we found that MO XMLP can significantly rescue the phenotype of apoptosis induced by *XMLP* (Fig. 6 B,C). No embryos out of 48 injected embryos showed apoptosis. 15 normal embryos and 15 eye-defect as well as mild bent axis defect out of 30 survived tadpoles were found in hatched tadpole stages (Fig. 6D and Table 1). The phenotype induced by MO XMLP was not found any more.

The glycine at position 2 and the PSD (ED) domain are correlated with apoptosis function in overexpression experiments

To gain insight into the role of conserved domains, we created four mutant constructs with PCR (Fig. 7): 1. change of glycine to alanine for mutant G2A in order to introduce adverse effect to the myristoylation consensus; 2. change of serine at position 83 to alanine for mutant S83A to prevent phosphorylation of serines in putative ED (PSD). (Phosphorylation plays a functional role in *in vitro* experiments of other members of MARCKS family); 3. deletion of the splicing domain from position 22 to 27 to yield XMLP SD deletion mutant; 4. deletion of K79 to S83 to yield XMLP ED deletion mutant containing truncated ED domain. The mRNAs of wild type and mutants were microinjected at different doses into two dorsal blastomeres of 4-cell stage embryos. We checked the effect at late neurula because apoptosis significantly starts at neurula stages in overexpression experiment (Fig. 8A to F and Table 2). For *XMLP*, the phenotype of apoptosis as described above was significant when 0.4 ng *XMLP* RNA was injected into embryos (Fig. 8A)(75 out of 85 embryos showed the phenotype). The mutants S83A, G2A, and ED, did not significantly cause

Fig. 4. RT-PCR of adult tissues with XMLP specific primers. *XMLP transcripts were seen in all tested tissues. However, lower abundance was detected in kidney and intestine. (br, brain; ey, eye; he, heart; in, intestine; ki, kidney; li, liver; mu, muscle; ov, ovary; sk, skin; sp, spleen; st, stomach; te, testis).*

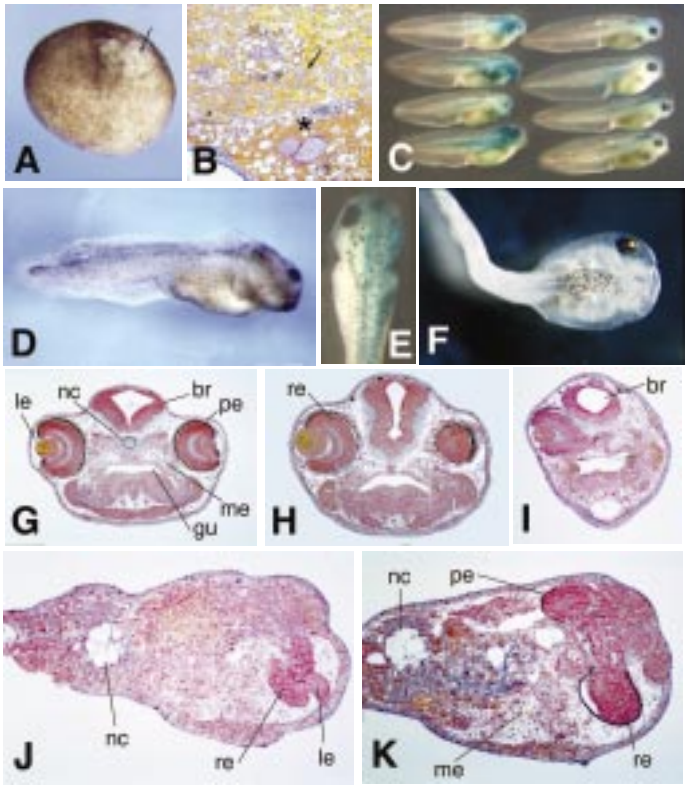

Fig. 5. The effect of overexpression with XMLP capped RNA. *Ectopic XMLP results in protrusions of bulge cells distributed around the injection site* **(A)**. **(B)** *Section of an XMLP-injected embryo at stage 10 as shown in (A). Note that there were some giant nuclei around the injection area (asterisk); a normal nucleus is indicted by an arrow. In addition, ectopic XMLP caused eye defects* **(C)** *where injected sides of the larvae were identified by LacZ as lineage marker. The larvae in the left column of panel (C) show smaller eyes at the injected side while the right column shows the unjected side of embryos of the same series (compare with larva shown from the dorsal side in* **(E)**). **(D)** *Larva (injected with* XMLP*) with bended axis and one eye.* **(E)** *Larva with missing right eye injected with both* XMLP *and LacZ into one dorsal blastomere of the 4-cell stage.* **(F)** *The injected larvae of stage 40 with one eye only. A comparison of the histology of notochord in the head area between normal larvae* **(G)** *and larvae with a small eye* **(H)**. *Note that less mesenchyme in (H) has been found in injected embryos compared to normal ones.* **(I)** *Transversal section of the tadpole with one eye shown in (F).* **(J,K)** *The differentiation of dorsal blastopore lip with adjacent dorsal ectoderm isolated from normal embryos (J) and injected embryos (K), respectively. Note that* XMLP*-treated embryos differentiated into notochord and brain structures with a rudimentary eye as the controls. No significant differences have been found in dorsal blastopore lips either isolated from injected or uninjected embryos (abbreviations: br, brain; bv, brain ventricle; gu, gut; le, lens; me, mesenchyme; nc, notochord; pe, pigmented epithelium; re, retina).*

apoptosis when injected corresponding mRNA with the same dose (10 of 85, 9 of 116 for G2A, 0 of 60, respectively), while all embryos (91of 91) injected with 0.4 ng mutant SD mRNA showed apoptosis. The results suggest that the mutants S83A, G2A and ED have reduced apoptosis inducing function in overexpression experiments. When the injected dose of S83A mRNA was doubled to 0.8 ng, 40 of 55 embryos showed the phenotype of apoptosis, in contrast only 5 out of 44 embryos injected with G2A and 0 of 59 embryos injected with ED mutant mRNA showed this phenotype.

In the case of mutant ED, only 7 of 69 displayed apoptosis after injection with 1.6 ng mRNA. The phenotypes of tadpole embryos after injection of the four mutants RNAs are shown in Fig. 8H to M and Table 2. The common features were eye defects, bent axis and in a few cases the severe reduction of the anterior axis. There was no significant difference between the mutants and *XMLP* with exception of apoptosis. However, when 1.6 ng ED mutant RNA was injected into two dorsal blastomeres of 4-cell stage, the ectoderm in the hind brain area of tailbud stage remained open, presumably due to an incomplete closure of neural folds (Fig. 8L). This phenotype is quite similar to F52 knockout mice, which showed exencephaly and postnatal anencephaly.

Ectopic XMLP expression blocks normal rhombomere formation

Whole-mount *in situ* hybridization indicated that *XMLP* transcripts were restricted to the presumptive neural system during neurulation. We therefore investigated the effect of ectopic *XMLP* expression on *Krox20*, a neural marker, whose expression is used to identify rhombomere 3 and 5 in the development of hindbrain (Fig. 9A). Overexpression of *XMLP* (capped *XMLP* mRNA synthesized *in vitro*) was carried out by injection into one dorsal blastomere of the 4-cell stage embryos employing *LacZ* as lineage tracer. Ectopic expression of the *XMLP* resulted in a decreased *Krox20* signal in the rhombomere 3 and 5 areas. The stripes looked irregular and even disappeared in 28 out of 48 cases on the injected side (Fig. 9B).

Retinoic acid hydroxylase (XCYP26) changed the expression pattern of XMLP

Retinoic acid (RA) is known to participate in the development of the nervous system and the formation of the limb buds (for review see Maden *et al.*, 1998; Zile, 1998; Hollemann *et al.,* 1998b). In order to test if the RA metabolites could affect the expression

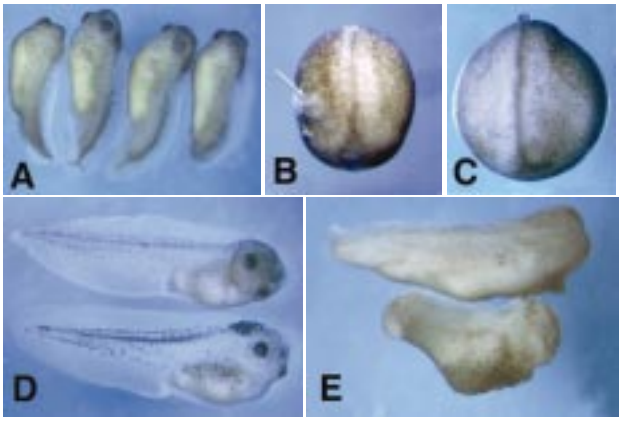

Fig. 6. Effect of Morpholino (MO) XMLP and MO β-catenin injected into embryos. (A) *24 ng MO XMLP was injected into two-cell stage embryos resulting in embryos with a shorter anterior axis and eye defects.* **(B)** *The phenotype of apoptosis induced by XMLP mRNA.* **(C)** *The phenotype of apoptosis can be rescued by MO XMLP (16 ng MO XMLP and 0.8 ng XMLP mRNA were injected simultaneously).* **(D)** *The injected tadpole with MO XMLP and XMLP mRNA showed a relatively normal morphological phenotype or a mildly bent axis (the upper one).* **(E)** *shows larvae with reduced anterior axis after microinjection of 4 ng of MO β-catenin into two dorsal animal blastomeres of 8-cell stage embryos.*

pattern of *XMLP* (Fig. 9C), stage 20 embryos were analyzed by whole-mount *in situ* hybridization with *XMLP* antisense RNA after co-injection of 2 ng *XCYP26* and 100 pg *LacZ* capped RNA into one blastomere of the 2-cell stage embryos. The results revealed that the signal stripes on the injected side were weaker or even disappeared (31of 54 cases) in contrast to the uninjected side (Fig. 9D).

Discussion

The present work shows that *XMLP* is involved in normal development of neural tube and the primordium of the central nervous system. It can also induce apoptosis in overexpression experiments. Data from other members of the MARCKS family together with our results suggest that *XMLP* is involved in morphogenetic movement during early embryonic development. The disturbance of morphogenetic movement and induced apoptosis by *XMLP* may be the reason for a reduction of the anterior axis and eye defects.

XMLP and XMARCKS show different temporal expression patterns

The temporal expression pattern of *XMLP* was different from its homologue *MARCKS* in *Xenopus*. For *XMARCKS*, there is a clear decreased level from 4-cell stage to mid blastula transition (MBT), which reflects the decay of maternal transcripts. Thereafter, zygotic transcription of the *MARCKS* appeared to be switched on and maintained permanently active throughout development (Shi *et al.*, 1997). In contrast the reduced level of *XMLP* is not as significant as that of *XMARCKS*, i.e. *XMLP* is expressed strongly at stage 8. The lowest expression level is reached at the early tailbud stage, followed by an increase of the *XMLP* expression and maintenance of a constant level. The differences between the two homologs may reflect the expressions of redundancies regulated by different mechanisms. The mice homologue of *XMLP* (F52) mRNA was found to be expressed at high level from 8.5 days post conception (dpc) through 14.5 dpc. At 17.5 dpc, the mRNA levels remained high in the brain only but not in other parts of the embryo. Thus, F52 mRNA is abundantly expressed during the period of neurulation (Wu *et al.*, 1996). Similarly *XMLP* is also abundantly expressed in the neural folds at neurula stage, and shows a high level of expression in the brain area of tadpole stage embryos.The signal location suggests that it play an important role in the development of the central nervous system.

TABLE 1

RESULTS OF INJECTION OF MOPHOLINO XMLP

MO or RNA injected	Injected blastomeres	phenotype of the embryos		
		Normal	Mild bent axis only	Shorten trunk, mild bent axis, eye defects, reduced head area
MO XMLP 16 ng	2/2	63 (46)	13 (9)	61(45)
MO XMLP 24 ng	2/2	0 (0)	7 (23)	23 (77)
MO XMLP 8 ng	2/4d	46 (66)	7 (10)	17 (24)
MO XMLP 16 ng	2/4 d	48 (62)	7 (9)	22 (29)
MO XMLP 24 ng	2/4d	4 (8)	0 (0)	47 (92)
XMLP 0.8 ng plus MO XMLP 16 ng	2/4d	15 (50)	15[a](50)	_

[a] The 15 injected tadpoles with *XMLP* 0.8 ng together with MO XMLP 16 ng showed lens defect and mild bent axis defect simultaneously.

The results for MO XMLP were scored when the embryos developed till around stage 40. 2/2 means that two blastomeres of 2-cell stage embryos were injected, 2/4d indicates two dorsal blastomeres of 4-cell stage embryos were injected. The percentage of the available cases is shown in parenthesis.

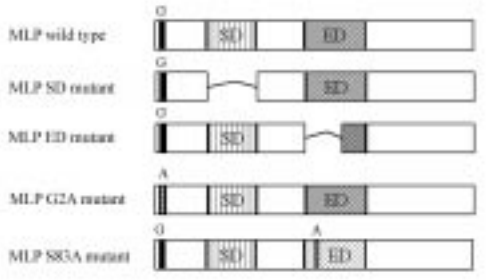

Fig. 7. Schematic drawing of XMLP and its mutant constructs used in this study. *Functional ED is indicated as a dark-slashed box, the site of intron splicing is indicated by vertical dashed box, and the glycine at position 2 is indicated by black box and the substituted alanine is shown by box with grid (A in the G2A mutant). The white-slashed box in the* XMLP *S83A mutant represents the mutant ED domain changing serine to alanine (A); the alanine is also shown by a box with grid.*

The blockage of XMLP can disturb normal anterior axis formation.

Embryos with blocked *XMLP* by injection of Morpholino (MO) XMLP showed head reduction and eye defects suggesting that *XMLP* be involved in anterior axis formation. The phenotype can be rescued by injection of *XMLP*, which indicates that MO XMLP specifically binds *XMLP* mRNA. Data from *in vitro* studies of other members of this protein family show that they are very important for cell shape and cell motility. MARCKS is found associated with the plasma membrane and in the cytosol. The translocation happened prior to changes in cell morphology (Ramsden, 2000). We suppose that the disturbance of normal morphogenetic movement cause the abnormal phenotype. If XMLP has a similar function as other members of this protein family, it is reasonable that the elimination of XMLP will change the cell morphology inevitably followed by a change of incident processes downstream in embryonic development.

The function of XMLP is correlated with its ED domain and glycine at position 2 - overexpression of XMLP can induce apoptosis

Apoptosis is a part of the developmental program of an organism. At the beginning we could not surely distinguish if the phenotype of overexpression experiments was due to RNA toxicity or specific function of *XMLP*. We therefore microinjected *LacZ* as control strictly under the same experimental conditions as *XMLP* capped mRNA. Since such phenotypes were not found in *LacZ* injected embryos, we could exclude the possibility of toxic effects (data not shown). The results from other authors also support our view in respect to this phenotype (Grammer *et al.,* 2000). Many cDNAs encoding factors which cause cell damage or inhibit normal cell cycle progression (transcription inhibitors, translation inhibitors, and mitosis blocking agents) can lead to the phenotype of apoptosis described above. Although there exist some reliable methods to show apoptosis in *Xenopus*, e.g. TUNEL or ELISA, it

would be important to focus the interest on apoptosis in overexpression experiments. This is beneficial in identification and characterization of cell death inducers and effectors, and the distinction of phenotype caused by direct inhibition or cell death.

Our results indicated that the ED domain (PSD) and the glycine at position 2 of the protein are important for the function properties. From our results, the substitute alanine for serine at the position 83 could reduce but not abolish *XMLP* apoptosis inducing activity in overexpression experiment because significant apoptosis was found when we increased the injected dose of S83A mRNA as shown in Table 2. However the intact ED domain is essential for this function. No significant differences were found between the SD mutant and *XMLP* wild type in overexpression experiments. But this highly conserved domain contained in MARCKS family must presumably have certain function though so far no experimental evidence exists. Overexpression of MARCKS in tumor-derived choroidal melanoma cells, where the amount of the endogenous protein was very low, could significantly decrease cell proliferation (Manenti *et al.*, 1998). Ectopic apoptosis may be one of the reasons for eye defects and reduction of anterior in overexpression experiments, since normal embryonic development could be inhibited by severe ectopic apoptosis (Grammer *et al.*, 2000). Overexpression of *XMLP* alone without any apoptosis does not inhibit the histolotypic differentiation of isolated dorsal blastopore lip.

Overexpression of mutant ED inhibited the closure of neural tube, but did not induce apoptosis

Interestingly embryos injected with 1.6 ng ED mutant mRNA showed a phenotype, which is quite similar to exencephaly in mice.

TABLE 2

COMPARISON OF THE EFFECT OF XMLP, ITS FOUR MUTANTS AND THE COMBINATION WITH MO XMLP

RNA injected		Normal (n)	Lens defect (n)	Axis defect (bent body axis) (n)	Phenotype showing lens defect, reduced anterior axis and bent axis defect simultaneously (n)	Apoptosis
XMLP	0.4 ng	0 (0)	0(0)	0 (0)	41 (100)	75 (88)
XMLP +MO XMLP	0.8 ng+16 ng	15(50)	0	0	15 (50)	0 (0)
ED	0.4 ng	29 (48)	21 (35)	10 (17)	0 (0)	0 (0)
	0. 8ng	5 (7)	39 (53)	12 (16)	18 (24)	0 (0)
	1.6 ng	23 (42)	0 (0)	3 (5)	29[a] (53)	7 (10)
G2A	0.4 ng	12 (23)	0 (0)	0 (0)	40 (77)	8 (7)
	0.8 ng	0	0 (0)	0 (0)	26 (100)	5 (11)
S83A	0.4 ng	16 (40)	8 (20)	16 (40)	0 (0)	10 (12)
	0.8 ng	4 (12)	0 (0)	0(0)	29 (88)	40 (73)
	1.6 ng	0 (0)	0(0)	1 (3)	31 (97)	48(98)
SD	0.2 ng	0 (0)	15 (45)	0 (0)	18 (55)	18 (49)
	0.4 ng	0 (0)	18 (30)	0 (0)	43 (70)	91(100)

[a] The phenotype is quite similar to exencephaly. The result was scored at middle tailbud stage when the eye rudiment has not yet clearly formed, so the embryos not showing exencephaly were scored

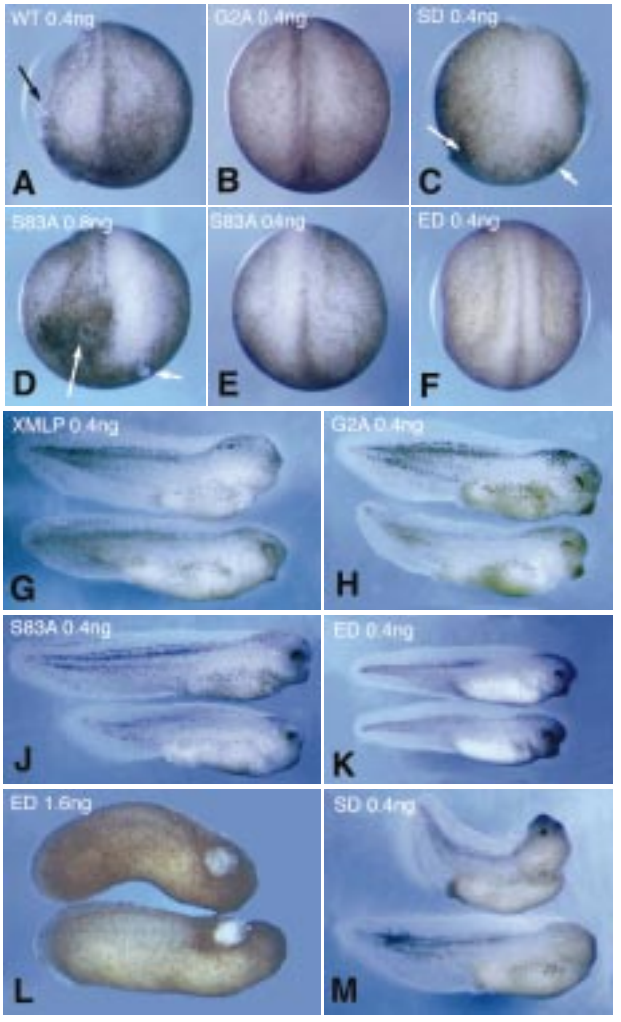

Fig. 8. Comparison of the phenotype induced by XMLP and its four mutants. *The phenotypes at neurula stage induced by 0.4 ng XMLP G2A, SD, S83A, ED are shown in* **(A)**, **(B)**, **(C)**, **(E)** *and* **(F)** *respectively. Note that apoptosis could be induced by 0.4 ng mRNA wild type of XMLP (A) and SD (C), but not by G2A (B), S83A (E) and ED (F). However the embryos showed apoptosis (D) when 0.8 ng S83A mRNA was injected to dorsal blastomeres of 4-cell stage embryos. The arrows in (A), (C), and (D) show the apoptosis area. The phenotypes in tadpole stages induced by G2A, SD, S83A, and ED with the dose of 0.4 ng mRNA are shown in (H) to (K) and (M). Larvae after injection of 0.4 ng XMLP are shown in* **(G)**. **(H), (J), (K),** *and* **(M)** *indicate the phenotypes induced by G2A, S83A, ED, and SD, respectively. The injected embryos showed eye defects and a reduced anterior region. (M) indicates the extreme case of injected embryos with SD mutant. Note that the phenotype shown in* **(L)**, *induced by 1.6 ng ED mRNA, is quite similar to the phenotype of exencephaly. The neural tube was not closed completely, and also the epidermis did not cover the internal yolk-rich yolk tissue.*

In contrast to wild type *XMLP* it did not cause apoptosis, but aberrant tissue morphogenesis including abnormalities in eye formation and neural tube closure. These data are in agreement with observations in the mice that knockout *XMLP* homologues led to embryonic exencephaly and postnatal anencephaly. Probably XMLP acts like F52, MARCKS and other PKC substrates such as GAP43 by mediating cytoskeletal changes and the control of morphogenesis in different tissues. We suppose that adverse ED

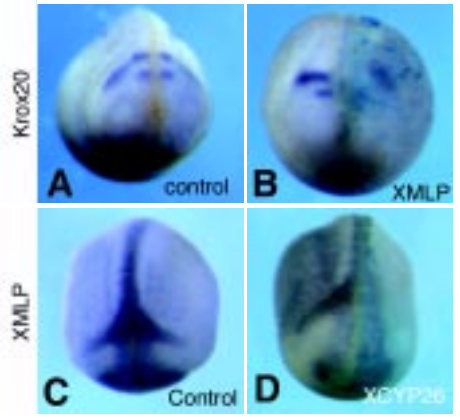

Fig. 9 Effect of ectopic XMLP on the expression of *Krox20* and the expression alternation of XMLP induced by XCPY26. *0.8 ng XMLP were injected into one dorsal blastomere of 4-cell stage embryos. β-gal encoding mRNA was co-injected as a lineage tracer (indicated by light blue). Signals were detected by whole-mount in situ hybridization with XMLP antisense RNA.* **(A)** *shows the expression of* Krox 20 *in the control embryos. The signal stripes of* Krox20 *at the injected side were disturbed* **(B)**. *2 ng XCYP26 capped mRNA was injected into one blastomere of 2-cell stage embryo which was visualized by LacZ staining (light blue stain). Signals were detected by whole-mount* in situ *hybridization with* XMLP *antisense RNA. The signal stripes that could be clearly seen on the normal embryos* **(C)** *vanished or got smeared* **(D)** *at the injected side indicated by LacZ.*

mutant molecules have a strong influence on cellular architecture. Introduced ED mutant molecules could compete with native XMLP *in vivo.* They could be adulterated the cellular architecture during embryonic development. Therefore they must presumably alter their cellular architecture, influence the motility, and alter their mutual cell affinity to the surrounding cells resulting in a failure of neural tube closure. In contrast MO XMLP did not cause such significant phenotype. This may be due to partial compensation by other redundant molecules. It is also possible that abundant XMLP was already present in the embryos when MO XMLP was injected. The data support the view that *XMLP* participates in morphogenetic movements. Morphogenesis including neurulation requires multiple cellular processes such as cell proliferation, cell shape change and cell migration (Schoenwolf, 1990). All these processes depend on the reorganization of the cytoskeleton, cell-cell signalling and mutual cell affinity. Ectopic expression of *XMLP* and morpholino injection had no obvious effects on morphogenetic movements during gastrulation since injected embryos showed normal blastopore and yolk plug formation. It could be argued that non-significant effects on embryonic development may partially correlated to still high levels of maternal *XMLP* transcripts and their long half-life.

Ectopic expression of XCPY26 affects the expression pattern of XMLP

Several experiments have shown that RA can alter the positional values of cells in limb development and in the anterior-posterior axis formation (Conlon, 1995). The effects of RA are mediated at different levels, but mainly by gene transcription and interactions with multiple nuclear receptors (RARS and RXRs). RAR-RXR heterodimer binds to RA response elements (RARES) in the promoter regions of target genes and regulate transcription in a ligand-dependent manner. XCYP26 is a RA hydroxylase, which could rescue RA induced developmental defect and alter the expression patterns of some other molecular markers for neural development (Hollemann *et al.*, 1998b). In our studies, we also found that *XCYP26* can influence the *XMLP* expression pattern. The signal stripe in stage 20 embryos on the injected side in contrast to that on the uninjected side gets diffused or even disappears. Therefore we suggest that RA participates in the regulation of *XMLP* and may be involved in anterior-posterior axis formation.

Materials and Methods

Isolation of XMLP

A ZAP Express phage cDNA library was constructed from activin-treated ectoderm (Cao *et al.,* 2001). Part of the phage cDNA library was converted into a plasmid library by *in vivo* excision according to the manufacturer's manual. The plasmid cDNA library was screened using large-scale whole-mount *in situ* hybrydization. Several interesting clones were selected for further functional study. *XMLP* is one of them.

Embryo manipulation

The eggs of *Xenopus* were obtained by injection of female frogs with 500-1000 IU human chorion gonadotropin (HCG) (Schering AG, Berlin) one night before artificial insemination. *In vitro* fertilization and embryo culture were carried out in 0.1×MBS. The jelly coat was removed prior to first cleave with 2% cysteine (pH 8.0). Embryos were staged according to Nieuwkoop and Faber (1975).

Plasmid construction

The predicted *XMLP* ORF sequence was subcloned into vector pCS2+ with PCR (Turner and Weintraub, 1994). *XMLP* G2A mutant were generated by primer *XMLP* G2A Fw: 5'GCGCGAATTCAATG**GCT**AGCGTAGAG3' (alanine codon is in bold) and *XMLP* G2A mutant Re: GCGCCTCGAGCTATTCCTCCTGTTT, changing glycine at position 2 to alanine. *XMLP* S83A mutant was obtained by amplifying plasmid *XMLP*-pCS2+ with a pair of 5'-phosphorylated primers: *XMLP* S83A Fw: 5'CTTGAA**AGC**GAACCTCTT 3' (alanine codon is in bold) and *XMLP* S83A Re: 5'AACCTGAAGTTCGGTAAT3' with long distance PCR, the PCR product was digested with DpnI to remove parental DNA and afterwards the resultant was blunt-end ligated with T4 DNA ligase. The supposed phosphorylation site serine at position 83 was replaced by alanine. For the *XMLP* ED mutant, the fragment of 5'ED was amplified *XMLP* subclone Fw: GCGCGAATTCAATGGGTAGCGT and 5'-phosphorylated ED primer 1: TGCCTGCTTTCCCGGTGG, and the fragment of 3'ED was amplified with 5'-phosphorylated ED primer 2: TTCAAGAACCTGAAGTTCGGTAAT and *XMLP* subclone Re: 5' GCGCCTCGAGCTATTCCTCCTG. Afterwards 5'ED and 3'ED were cut by *Eco*RI and *Xho*I respectively, and then coligated with *Eco*RI - *Xho*I- digested pCS2+. For *XMLP* SD mutant, the subclone was performed in two steps. 5'SD was amplified from *XMLP*-pbk-CMV containing full sequence of *XMLP* with two primers: CMV-F: CGCGCCTGCAGGTCGACACTA and 5'- phosphorylated SD mutant primer 2: TTGGTCTGCCTGCTTGTTGCT, in turn was cut by *Eco*RI and gel purified. 3'SD was amplified with SD mutant primer 3: 5'-phosphorylated AAAACCAATGGCGACGCCCCC and *XMLP* subclone Re, cut by *Xho*I, gel purified. The 5'SD and 3'SD were coligated with *Eco*RI-*Xho*I- cut pCS2+. Afterwards the resultant was amplified with *XMLP* subclone Fw and *XMLP* subclone Re, and subcloned into pCS2+.

Morpholino-Oligos and mRNAs

After linerization with *Not*I, the DNA templates were transcribed *in vitro* with *SP6* RNA polymease in the presence of m7GpppG to produce capped *XMLP* and mutant mRNAs using *SP6* Scribe (Roche, Mannheim, DE).

The antisense morpholino oligo used was a 25-mer-morpholino oligo (Gene Tools, Corvallis, USA) with the base composition of *XMLP*:

GGACTCTACGCTACCCATTGTGACT. *Xenopus laevis* ß-catenin: TTTCAACCGTTTCCAAAGAACCAGG was used as positive control. Morpholino oligos were resuspended in sterile milipore water, diluted with 1 x MBS (88 mM NaCl, 1 mM KCl, 0.7 mM $CaCl_2$, 1 mM $MgSO_4$, 5 mM HEPES (pH 7.4), 2.5 mM $NaHCO_3$) to certain concentrations. No significant toxic effect was found by the injection of the negative control: CCTCTTACCTCAGTTACAATTTATA.

Isolation of RNA and semiquantitive RT-PCR

The total RNA from embryos of *Xenopus laevis* and animal caps were isolated with RNA Clean™ (Hybaid-AGS, UK). The total RNA from adult tissue was extracted with phenol/chloroform and LiCl precipitation (Döring and Stick, 1990). 1 µg RNA was reversed transcribed by reverse transcriptase (SuperScript™ from GibcoBRL, USA) as described in the manufacturer's protocol. To get accurate quantitative results, multiplex PCR was performed to analyze mRNA level, i.e., briefly the primer of testing gene and internal standard control, respectively, were introduced in the same reaction tube early or late according to its optimal cycle number. 20 µl PCR product from 25 µl PCR reaction system was finally loaded on normal agarose gel for detection and results were determined by UV illumination. Each primer pair has been tested for primer-primer interactions before actual PCR reaction. The following primers were used:
XMLP (270 bp) forward: 5' GTCTAATGGCTCCGCTGA AG 3' and reverse: 5' GCTTCTGGAGATGCTTCCAC 3';
ODC (132 bp) forward: 5' GGAGCTGCAAGTTGGAGA 3' and reverse: 5' ATCAGTTGCCAGTGTGGTC 3';
H4 (188 bp) forward: 5' CGGGATAACATTCAGGGT A 3' and reverse: 5' TCCATGGCGGTAACTGTC 3'.

Whole-mount* in situ *hybridization

Whole-mount *in situ* hybridization was performed as described elsewhere (Harland, 1991; Oschwald *et al.*, 1991) with modifications as reported by Hollemann *et al.* (1998a). Dig-labelled antisense probe was synthesized using RNA polymerase and DIG RNA labelling Kit (Roche, Mannheim) after linearization.

LacZ staining

Embryos were fixed in HEMFA for 1 h and then rinsed in PBS 2-3 times, each for 10 min. Color reaction was performed in staining solution (β-galactosidase, 1 mg/ml, $K_3Fe(CN)_6$ 5 mM, $K_4Fe(CN)_6$ 5 mM, $MgCl_2$ 2 mM in PBS) for 1-6 h at room temperature. After being rinsed twice in PBS embryos were fixed again in HEMFA for 1 h, rinsed in 100% ethanol and stored at –20°C prior to whole-mount *in situ* hybridization.

Microinjection

For injection of *XMLP* mRNA, dejelled embryos were placed in 4% Ficoll400 dissolved in 1 x MBS Embryos were injected at 2-cell or 4-cell stage and subsequently kept in the same solution for one hour after injection. Then they were transferred to Holtfreter solution (60 mM NaCl, 0.6 mM KCl, 0.9 mM $CaCl_2$, 2.5 mM $NaHCO_3$, 5 mM HEPES (pH7.4)) for further development. In control series *Lac Z* capped mRNA alone was injected into embryos. *XMLP* and its mutant mRNAs either alone or together with 100 ng *lacZ* were injected into dorsal blastomeres of 4-cell stage embryos. 2.0 ng *XCPY26* capped mRNA (Hollemann, *et al.,* 1998b) prepared as above was injected into one blastomere of 2-cell stage embryos with *LacZ* mRNA for tracing cell lineage. Morpholino oligos diluted in 1 x MBS with different concentrations were injected into embryos respectively.

Histology

Standard techniques were used to prepare histological sections as described (Grunz, 1977, 1983). Embryos after whole-mount *in situ* hybridization were sectioned (12 µm thickness) and counterstained with eosine.

Data documentation

Macroscopic and microscopic documentation were performed with a Zeiss -Stereomicroscope and a Zeiss Axioplan-microscope respectively, and recorded with a SONY progressive 3 digital camera and a Zeiss MC100 camera. Sections were photographed with a Leitz-Orthomat-camera using Kodak Ektachrome 64T professional film. Slides were scanned with a Nikon-LS-1000 for further processing with Adobe Photoshop 6.0.

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