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 morphogenetic 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 diacylglycerol-activated and Ca²⁺-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; Blakshear, 1993).

MARCKS behaves as a PKC substrate and binds to calmodulin in a Ca²⁺-dependant manner. However, there is no direct evidence showing that the protein contains a Ca²⁺ 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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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 like protein (XMLP) (Fig. 1) has been reported in mouse embryonic carcinoma lines (Blackshear, 1993).

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 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 79 surrounding the site of intron splicing is also found in other members of the MARCKS family. The domain from K78 to K99 is the putative PSD domain. The three conserved sites mentioned above are underlined.

**Results**

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

Large scale screening of a cDNA library prepared from activin-treated oocyte 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 other species. One of them composed of 187 predicted amino acids was related to 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 laminar abnormalities of the cortex and retina. But by expression of non-mystostylated 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 oocyte cDNA library. We found XMLP was differentially expressed during early *Xenopus* development. Ecotopic 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 *XCMF* indicating that retinoic acid (RA) may play an important role for its regulation.
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).

**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 membrane 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...
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).

**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 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 lip from un.injected 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 strongly 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 nuclease (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 morpholino 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 β-catenin MO at 8-cell stage showed a reduction of the head area and anterior axis (Fig. 6E). Injection of 8 ng MO β-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 functional PSD domain; 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...
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

apoptosis when injected corresponding mRNA with the same dose (10 of 85, 9 of 116 for G2A, 0 of 60, respectively), while all embryos (91 of 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.
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 (31 of 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.

**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.

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### TABLE 2

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

<table>
<thead>
<tr>
<th>RNA injected</th>
<th>Normal Lens defect</th>
<th>Axis defect (bent body axis)</th>
<th>Phenotype showing lens defect, reduced anterior axis and bent axis defect simultaneously</th>
<th>Apoptosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>XMLP 0.4 ng</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>41 (100)</td>
<td>75 (88)</td>
</tr>
<tr>
<td>XMLP + MO XMLP 0.8 ng+16 ng</td>
<td>15 (50)</td>
<td>0 (0)</td>
<td>15 (50)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>ED 0.4 ng</td>
<td>29 (48)</td>
<td>21 (35)</td>
<td>10 (17)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>0.8 ng</td>
<td>5 (7)</td>
<td>39 (53)</td>
<td>12 (16)</td>
<td>18 (24)</td>
</tr>
<tr>
<td>1.6 ng</td>
<td>23 (42)</td>
<td>0 (0)</td>
<td>3 (5)</td>
<td>29 (53)</td>
</tr>
<tr>
<td>G2A 0.4 ng</td>
<td>12 (23)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>40 (77)</td>
</tr>
<tr>
<td>0.8 ng</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>26 (100)</td>
</tr>
<tr>
<td>S83A 0.4 ng</td>
<td>16 (40)</td>
<td>8 (20)</td>
<td>16 (40)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>0.8 ng</td>
<td>4 (12)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>29 (88)</td>
</tr>
<tr>
<td>1.6 ng</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>1 (3)</td>
<td>31 (57)</td>
</tr>
<tr>
<td>SD 0.2 ng</td>
<td>0 (0)</td>
<td>15 (45)</td>
<td>0 (0)</td>
<td>18 (55)</td>
</tr>
<tr>
<td>0.4 ng</td>
<td>0 (0)</td>
<td>18 (30)</td>
<td>0 (0)</td>
<td>43 (70)</td>
</tr>
</tbody>
</table>

*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.

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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 XMLPhomologues 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 alteration 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 Krox20 in the control embryos. The signal stripes of Krox20 at the injected side were disturbed (B). 2 ng XCPY26 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.

The data support the view that XMLP participates in morphogenesis including neurulation requires the expression alternation of XMLP induced by XCPY26.

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.

XCPY26 is a RA hydroxylase, which could rescue RA induced developmental defect and alter the expression patterns of other molecular markers for neural development (Hollemann et al., 1998b). In our studies, we also found that XCPY26 can influence the XMLP expression pattern. The signal stripe in stage 20 embryos on the injected side in contrast to that on the un.injected 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 ecdetorm (Cao et al., 2001). Part of the phage cDNA library was converted into a plasmid library by in vitro excision according to the manufacturer’s manual. The plasmid cDNA library was screened using large-scale whole-mount in situ hybridization. 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) (Scherering 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 was generated by primer XMLP G2A Fw: 5’GCGCGATATCAATGCGCTAGCGCTAGAG3’ (alanine codon is in bold) and XMLP G2A mutant Re: GGCCTCGACGTATTCCTCTGTTTT, 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’CTGCGAAGCAGACCTTCGT3’ (alanine codon is in bold) and XMLP S83A Re: 5’ACCCTGAGTTCCGATAT3’ 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 S83A mutant, the fragment of 5’ED was amplified XMLP subclone Fw: GGCCTCGACGTATTCCTCTGT and 5’-phosphorylated ED primer 1: TGCCCTGCTTTCCCGTG, and the fragment of 3’ED was amplified with 5’-phosphorylated ED primer 2: TTAAGAACTCTGAGTCTGAT and XMLP subclone Re: 5’GCCCTCGAGCTATTCCTCTCTAG. Afterward the resultant was amplified with EcoRI and XhoI and then digested with EcoRI and gel purified. The 5’ and 3’SD were amplified from XMLP-pbk-CMV containing full sequence of XMLP with two primers: CMV-F: CGGCGCTGAGGATACACACTA and 5’-phosphorylated SD mutant primer 2: TTGAGCTGGCGCTGTGTGCT, in turn was cut by EcoRI and gel purified. The 5’ and 3’SD were subcloned into pCS2+ vector. Afterwards the resultant was amplified with XMLP subclone Fw and XMLP subclone Re, and subcloned into pCS2+ vector.

Morpholino-Oligos and mRNAs

After linearization with NotI, 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:
GGACTCTAGCTACCCATTTGACT. *Xenopus laevis* B-catenin: TTCACACGTCTCTCCAGGAGGG was used as positive control. Morpholino oligos were resuspended in sterile milipore water, diluted with 1× MBS (88 mM NaCl, 1 mM KCl, 0.7 mM CaCl₂, 1 mM MgSO₄, 5 mM HEPES (pH 7.4), 2.5 mM NaHCO₃) to certain concentrations. No significant toxic effect was found by the injection of the negative control: CTCCTACGTCTAGTTAATAG.

**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’ TCTTACATCTCCGCTGTA 3’ and reverse: 5’ GCCTCTGGAAGATGCTCCAC 3’; ODC (132 bp) forward: 5’ GGAGCTGCAATTTGAGA 3’ and reverse: 5’ ATCAGTGGCAATTTGCTC 3’; H4 (188 bp) forward: 5’ CGGCGATCTCAAGGGC 3’ and reverse: 5’ TCCATGGGCTTAACTGC 3’.

**Whole-mount in situ hybridization**

Whole-mount *in situ* hybridization was performed as described elsewhere (Harland, 1991; Oszwald et al., 1991) with modifications as reported by Hollemann et al. (1998a). Dig-labeled 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, K₃Fe(CN)₆ 5 mM, K₄Fe(CN)₆ 5 mM, MgCl₂ 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 CaCl₂, 2.5 mM NaHCO₃, 5 mM HEPES (pH7.4)) for further development. In control series LacZ 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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