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Expression of *Panza*, an $\alpha 2$ -Macroglobulin, in a Restricted Dorsal Domain of the Primitive Gut in *Xenopus laevis*

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Abstract

$\alpha 2$ -macroglobulin is a major serum protein with diverse functions, including inhibition of protease activity and binding of growth factors, cytokines, and disease factors. We have cloned and characterized *Panza*, a new *Xenopus laevis* $\alpha 2$ -macroglobulin. *Panza* has 56–60% amino acid similarity with previously identified *Xenopus*, mouse, rat and human $\alpha 2$ -macroglobulins, indicating that *Panza* is a new member of the $\alpha 2$ -macroglobulin family. *Panza* mRNA is first detected at the beginning of neurulation in the dorsal endoderm lining the primitive gut (archenteron roof). At the completion of neurulation and continuing through the late tadpole stage, *Panza* is restricted to a dorsal domain of the gut endoderm adjacent to the notochord and extending along the entire anterior-posterior axis. With outgrowth of the tailbud, *Panza* expression persists in the chordaneural hinge at the posterior end of the differentiating notochord and extends into the floor plate of the posterior neural tube. As gut coiling commences, *Panza* expression is initiated in the liver, and the dorsal domain of *Panza* expression becomes limited to the midgut and hindgut. With further gut coiling, strong *Panza* expression persists in the liver, but is lost from other regions of the gut. The expression of *Panza* in endodermal cells adjacent to the notochord points to a potential role for *Panza* in signal modulation and/or morphogenesis of the primitive gut.

Keywords

Xenopus laevis; $\alpha 2$ -macroglobulin; endoderm; gut; digestive tract; liver

1. Results and Discussion

$\alpha 2$ -macroglobulin ($\alpha 2$ M) is an abundant plasma protein of vertebrates and arthropods that has a remarkable capacity to bind numerous and diverse ligands. $\alpha 2$ M was first identified as a “panprotease inhibitor” capable of binding nearly all extracellular proteases, leading to clearance and degradation of $\alpha 2$ M-protease complexes. Binding of protease to native $\alpha 2$ M results in cleavage and activation of $\alpha 2$ M, causing a conformational change that entraps protease and exposes binding sites for the $\alpha 2$ M receptor. The $\alpha 2$ M-protease complex binds to low density lipoprotein receptor-related protein (LRP), the major cell surface receptor for $\alpha 2$ M, and the receptor bound complex is internalized by endocytosis, and targeted for lysosomal degradation (reviewed in Borth, 1992).

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The conformational change that occurs with α_2M activation also exposes binding sites for non-protease ligands, including PDGF (Huang et al., 1984), FGF (Mathew et al., 2003), TGF β 1 (Huang et al., 1988; Stouffer et al., 1993; Feige et al., 1996; Arandjelovic et al., 2003), Activin A (Niemuller et al., 1995; Mather, 1996; Phillips, 2000), NGF (Ronne et al., 1979), TNF (James et al., 1992) and multiple Interleukins (Borth and Luger, 1989; Borth et al., 1990; Garber et al., 2000; Kurdowska et al., 2000). In complex with these ligands, α_2M can serve either as a carrier that stabilizes ligand in circulation, a clearance factor for ligand degradation, or as a targeting factor for cellular uptake and subcellular localization of ligand. For example, native α_2M is an Activin A carrier that stabilizes circulating levels, while activated α_2M clears circulating Activin A via LRP-mediated internalization (Niemuller et al., 1995). The ability of α_2M to bind and regulate such a diverse group of ligands suggests that α_2M may modulate multiple signaling and morphogenetic processes in the embryo and adult.

A role for α_2M in Alzheimer's disease (AD) was first suggested by the localization of α_2M to senile plaques of AD patients (Van Gool et al., 1993). Activated α_2M binds to β -amyloid (A β) and the α_2M -A β complex is cleared and degraded by LRP-mediated internalization (Narita et al., 1997). Consistent with a role in regulating A β function, α_2M reduced A β aggregation and fibril formation, as well as the resulting neurotoxicity in cortical neurons (Du et al., 1998; Hughes et al., 1998). The importance of the α_2M -A β interaction in AD is supported by the strong genetic association of multiple α_2M polymorphisms with increased accumulation of A β plaques in the brains of late-onset AD patients (Kovacs, 2000; Saunders et al., 2003). Interestingly, the α_2M polymorphisms associated with AD do not appear to result in a reduction of α_2M levels, and human subjects with heterozygous deletion of α_2M have no apparent clinical symptoms (Bergqvist and Nilsson, 1979). Instead, the α_2M polymorphisms are predicted to stabilize α_2M mRNA or result in a truncated α_2M protein that binds A β but cannot be internalized via LRP (Kovacs, 2000).

In mammals, the liver is the principal site of α_2M expression with onset at mid-gestation and continued expression in the adult. Additional major sites of α_2M expression include the brain, spinal cord, and eye (Kodelja et al., 1986; Lorent et al., 1994), and expression is also detected in testis, ovary, uterus, and placenta (Hayashida et al., 1986; Northemann et al., 1988; Gaddy-Kurten et al., 1989; Cheng et al., 1990). α_2M -deficient mice are viable and fertile, and have no apparent embryonic or adult phenotype other than slightly altered toxin sensitivity (Umans et al., 1995). However, *murinoglobulin*, an α_2M -related gene, is strongly upregulated in α_2M null mice and may compensate for the loss of α_2M function. Compensation by upregulation of α_2M -related genes may also account for the absence of symptoms in human subjects with heterozygous deletion of α_2M .

In *Xenopus laevis*, a single α_2M gene, *Endodermin* (*Edd*), was previously isolated in a differential screen for genes expressed in dorsal cells of the gastrula (Sasai et al., 1996). *Edd* is first expressed in endoderm and dorsal mesoderm at the gastrula stage, and at the neurula stage, *Edd* is expressed in the notochord, prechordal plate, hatching gland and throughout the endoderm. At the tailbud stage, high *Edd* expression is detected throughout the endoderm, with weaker expression in the notochord and otic vesicle. With the onset of gut coiling, *Edd* expression persists in liver, but is lost from other tissues (Sasai et al., 1996; Chalmers and Slack, 1998; Zorn and Mason, 2001). Here we report the identification of *Panza*, a second *Xenopus* α_2M gene. Unlike *Edd*, *Panza* expression is limited to a dorsal domain of the gut endoderm adjacent to the notochord, an expression pattern suggesting a potential role for *Panza* in signal modulation and/or morphogenesis of the primitive gut.

To identify additional α_2M genes in *Xenopus*, a degenerate PCR strategy was pursued using primers corresponding to highly conserved regions of α_2M orthologs. A product with high similarity to α_2M was obtained from tailbud stage cDNA, and 5'/3' RACE was subsequently

used to isolate a complete open reading frame. Conceptual translation yielded a protein of 1474 residues containing an N-terminal signal sequence, a bait region, and a thioester site, which are features characteristic of α_2 M proteins (Fig. 1A) (Borth, 1992). This sequence has strong amino acid similarity (56–60%) with Endodermin, and the mouse, rat and human α_2 M proteins (Fig. 1B), indicating that it is a new member of the α_2 M family. We have named this gene *Panza*, from the Spanish for gut or belly (accession number [DQ080115](#)).

The temporal expression pattern of *Panza* was examined by RT-PCR analysis of staged embryos (Fig. 1C). *Panza* was not detected in pre-MBT samples (stages 0–8), indicating that it is not expressed maternally. Zygotic expression of *Panza* is initiated at the early neurula stage (stage 13) and continued expression is detected in all subsequent stages through the swimming tadpole stage (stage 45). In comparison, *Edd* expression is initiated earlier, at the onset of gastrulation (stage 10), and continues in all stages examined.

The spatial expression pattern of *Panza* was determined by whole-mount in situ hybridization (Fig. 2). *Panza* expression was first detected at the early neurula stage in the archenteron roof, the endoderm forming the dorsal surface of the primitive gut. *Panza* extends along the midline of the anterior-posterior axis from the oral evagination to the blastopore, with expression more confined to the midline in the anterior gut and extending more laterally in the posterior gut. *Panza* is also expressed at the midline of the posterior neural plate and, with the completion of neurulation, in the floor plate of the posterior neural tube. These features of *Panza* expression are maintained during axial elongation and through the onset of gut coiling (stages 14–40) (Fig. 2A,C,E,G,I and data not shown). In the tailbud, *Panza* expression extends dorsally around the posterior chordamesoderm and into the floor plate of the posterior neural tube, an expression domain corresponding to the chordaneural hinge. With tailbud outgrowth, *Panza* expression is maintained around the tip of the posterior notochord and in the floorplate of the posterior spinal cord (Fig. 2I and Fig. 3). In contrast, *Edd* is expressed throughout the dorsal, lateral and ventral endoderm at neurula stages and later, with weaker expression in the notochord, otic vesicle, and hatching gland, but not in the neural tube (Fig. 2B,D,F,H,J) (Sasai et al., 1996; Chalmers and Slack, 1998; Zorn and Mason, 2001).

Given the juxtaposition of *Panza* with chordamesoderm and notochord during axial and tailbud elongation, we examined the spatial relation of *Panza* expression with the notochord (Fig. 3). Embryos were sequentially stained in whole-mount for *Panza* by in situ hybridization and for notochord by immunohistochemistry with the Tor70 antibody (Bolce et al., 1992). Throughout much of the body axis, *Panza* is expressed by endodermal cells subadjacent to the notochord, with *Panza*-positive cells either in direct contact with the notochord, or close to it (Fig. 3A,A1–A4). *Panza* is not expressed by cells of the notochord. In addition, *Panza* expression is not detected in the hypochord, a structure positioned at the midline between the notochord and gut (Fig. 3B). At the level of the eyes, anterior to the tip of the notochord, *Panza* is expressed in the dorsal endoderm of the oral evagination, adjacent to the ventral surface of the brain (Fig. 3A1). At the level of the anterior trunk, *Panza* expression is limited to endodermal cells in close proximity with the notochord (Fig. 3A2), while in the posterior trunk, *Panza* expression extends further from the notochord, but with higher levels in endodermal cells adjacent to the notochord (Fig. 3A3). At the level of the tailbud, *Panza* expression extends even further to the lateral endoderm of the gut, and is also expressed in the floorplate domain of the posterior spinal cord (Fig. 3A4). With tailbud extension, *Panza* expression is maintained in cells ventral to the notochord, in the chordaneural hinge at the posterior tip of the notochord, and in the floorplate of the posterior neural tube (Fig. 3C–E).

To examine *Panza* expression at later stages when the process of gut coiling is initiated, gut structures dissected from swimming stage tadpoles (stages 40–46) were subjected to whole-mount in situ hybridization (Fig. 4). At the onset of gut coiling, *Panza* expression is restricted

to the dorsal midgut and hindgut, with broader and more intense expression in the hindgut (Fig. 4A,A'). As coiling progresses, *Panza* expression is maintained in a subset of midgut cells, spreads to encompass the entire hindgut, and is initiated in the liver (Fig. 4C,C',E,E',G,G'). With further gut coiling (stage 46), *Panza* expression is lost from all regions of the gut except the liver, where strong expression is maintained (Fig. 4I). No *Panza* expression is observed in the gallbladder, pancreas or stomach at any stage examined (see Fig. 4G). In contrast to *Panza*, *Edd* is expressed in the liver and throughout the midgut and hindgut from stages 40–44 (Fig. 4B,D,F,H). By stage 46, *Edd* is lost from all gut regions and organs except the liver (Fig. 4J).

In summary, *Panza* marks a unique domain of the primitive gut from the earliest stages of gut development. Taken together, the presence of *Panza* in endodermal cells adjacent to the notochord and the ability of α_2 M proteins to regulate the activity of multiple proteases and signaling factors, point to a role for *Panza* in patterning, differentiation and/or morphogenesis of the primitive gut in *Xenopus*.

2. Experimental procedures

2.1 *Xenopus* embryos

Xenopus laevis embryos were obtained using standard procedures (Sive et al., 2000) and staged according to the normal table of *Xenopus* development (Nieuwkoop and Faber, 1967).

2.2 Cloning of *Panza*

Degenerate primers were designed based on two protein sequences, PETWIWD and GCGEQNM (residues 748–754 and 983–989 of mouse α_2 M), conserved in multiple α_2 M orthologs (Fig. 1A). The upstream (5'-CCIGARACITGGATHTGGGA-3') and downstream (5'-CATRTTYTGYTCICCRCAICC-3') primers were used to amplify α_2 M-related sequences by RT-PCR of tadpole total RNA (stage 35). A major product of the predicted size (~730 bp) was obtained, individual subclones were sequenced, and multiple isolates of *Panza* were identified. The remainder of the *Panza* open reading frame was isolated using the 5'/3' SMART RACE cDNA amplification kit (Promega). A single round of 3' RACE primed with 5'-ACAGCCATGCAGAACCTGGACCGT-3' yielded the complete 3' end of the mRNA, and two rounds of 5' RACE primed with 5'-CCCTCCGCTCTGGATAGAAACCTGC-3' and 5'-CCCTCCGCTCTGGATAGAAACCTGC-3' yielded the 5' end of the open reading frame. The sequence of *Panza* has been deposited in GenBank (accession number [DQ080115](#)).

2.3 RT-PCR

Total RNA was isolated using the RNAqueous kit (Ambion) and RT-PCR was performed as described previously (Wilson and Melton, 1994). *Panza* primers were 5'-TCTAACCTGGGAGAGGTGAA-3' and 5'-TATGTCTCCAAGGACCGTCA-3', Endodermin primers were 5'-TATTTGACTCCTGAAGGTG-3' and 5'-GAGAACTGCCCATGTGCCTC-3', and ornithine decarboxylase primers were as described (Agius et al., 2000).

2.4 In situ hybridization, Immunohistochemistry and Histology

Whole-mount in situ hybridization was performed as previously described (Sive et al., 2000). Digoxigenin-labeled antisense probes for *Panza* and *Endodermin* were synthesized from linearized pBluescript-*Panza* and pBluescript-*Edd* (Sasai et al., 1996) using the Megascript kit (Ambion). For in situ hybridization of isolated *Xenopus* guts, anesthetized tadpoles (stages 40–47) were placed in MEMFA fixative, guts were removed with fine forceps, and the exposed guts were fixed for 45 minutes. In some cases coiled guts were straightened and held under

parafilm while in fixative. For detection of notochord following in situ hybridization, post-fixed embryos were processed for whole-mount immunohistochemistry as previously described (Sive et al., 2000). Notochord was detected with Tor70 antibody (Bolce et al., 1992) at 1:300 dilution, HRP-conjugated rabbit-anti-mouse Fab₂ (Jackson Immunoresearch) at 1:250 dilution, and was visualized with DAB. For photography, embryos were dehydrated and cleared in benzyl benzoate/benzyl alcohol. Embryos double-stained for *Panza* and notochord were processed for histological analysis using standard methods (Sive et al., 2000), and dewaxed sections (18 µm) were coverslipped with Permount without counterstaining.

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1  MLLRVLHVGL LLLQLSGTPE AKLQYAVIFP AEMVSPHSEQ ACVHLEGANG 50
51  ESKIQLTLQM AKSNTTVEIK TSEQKSYFRC VSFEVPPPSE ENEEVATMQV 100
101 SIQSGGETIS NSSKVLVKKK RTSIFVQTDK AIYKPGQTVR FRVVSLEKENL 150
151 QPEESQVPTI ELQDPGKNRI GQWLVNLSIQ GIAELSLPLS SEPPLGEYSI 200
201 HVKNTVHVFS VEEYVLPFE VTLQLPKVVV VVTGQFPVKI CARYTYGKPV 250
251 QGTYKVDVCR KHSYRRAFG NDKGRDLCAD FSGKLDQSGC STFDVKSSNY 300
301 NLLSSEWQMN IEGAGWITED GTGIQISATD KSQISYDLTS VTFLDADDTY 350
351 KPGIPYIGTV KVDASDTPI PKHPVYLIDT DGIVNMTLMT DKNGLAPFKL 400
401 DNTADWKATV SLTAKTQVKE PLMQYQYGM FPRHGLAMLR LNRFYSRSKS 450
451 QKYLHSQNGE FLCEGQQEVQ VEYLKRTTEL GEEAKHLELH YLAVSKGAIK 500
501 ESDVLEVPIN NDGEVLRGEV SIKLKLCDVD SPTLRVLTIV LLPGGEMVAD 550
551 AAKFKLQKCF RNKVSVGFSF DEVLPGSDVS LQVQAAAGSL CGLRVVDKSV 600
601 VLMKPDQELT ADKIRGLFPV NDFGNYDYRI QEAEHDCPFR PFLPWRPFNR 650
651 ISRSIIYPPW HMPPEADVYG LFKKVQLKII TSADIKKPVV CQHYDFLEPG 700
701 LKGLPGFGD RMIAFASPGI SAAGSIPNQL PEAEAAVEI IRTYFPETWI 750
751 WELASVGESG TAEHLRAVPD TITDWNAGAF CMGPGSGFGIS SPTSLRVFPQ 800
801 FFEVITLPS VVRGESFALK ATVFNYLKQC IKVQTALEPS QELEEEPCPD 850
851 CQYSSICAE ESKTSYWNVK ASNLGEVNV AKTAALDQD LCNNEIPIVP 900
901 KQGRSDTVIK PLLLQPGGV LEEKAHSSLLC CQAGDDHPKT EEISLKVPE 950
951 ILKDSERAYV TVLGDIMGTA MQNLDRLLAM PYGCEGQNMV LFAPNIFILQ 1000
1001 YLEKTHQLSP EIQSKAKRFL ESGLRQLTY KRDDGSYSF QQRDKEGNTW 1050
1051 LTAFFVKSFS KAPYIFIDE SHLKHSFTWL KNNQHEGWF QSVGRLEFNA 1100
1101 MKGGIDDETS LSTYVTMALL EAGVSVQNPV VADALSCLRK AAIDVSSVYT 1150
1151 QALLAYTFTL SDDTELREML LAKLEEKAVR NEGQLHWERK PATAPSDLSY 1200
1201 WSRAPSAEVE LTSYVLLALL SGPKNKDLGKA SEIVNWLSKQ QNPYGGFSST 1250
1251 QDTVVALLQAL AKYAEATFTD KGDVTVTVSS KTGFGHQFHV DHTNRLLLQK 1300
1301 SSSLDIPGEF SLSVTGSGCV YIQTVLRYNI PPPRSDATFS VRVETQPDKC 1350
1351 PQHPLEVIKI NITAEYTGSR EKSNAVIEC KMLSGFIPVK SSVRELEKSK 1400
1401 TIKRSEIQT DMTLYLDELG HEPLNLFETV EQDIVVKNIK PATVKVYDYY 1450
1451 ETGEEAVAEY NSPCSSDEEK GNTR 1474

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B

	X PANZA	X EDD	M α 2M	R α 2M	H α 2M
X PANZA	100	59	56	59	60
X EDD		100	62	64	66
M α 2M			100	69	71
R α 2M				100	83
H α 2M					100

Figure 1.

Xenopus Panza is a new member of the α 2-macroglobulin family. (A) Amino acid sequence of *Panza* with the predicted signal sequence (1–21), bait region (668–708), thioester site (984–987), and peptide sequences corresponding to the degenerate primers (arrows above sequence) indicated. (B) Relatedness of *Panza* to other α 2M proteins. Percent amino acid similarity is presented for *Xenopus* Endodermin (X EDD; L63543), and mouse (M α 2M; M93264), rat (R α 2M; J02635) and human (H α 2M; M11313) α 2-macroglobulin. (C) Temporal expression pattern of *Panza* in comparison with *Endodermin* (Edd). RT-PCR analysis was performed on total RNA isolated from embryos at the indicated stages. *Ornithine decarboxylase* (ODC)

served as a control for RNA recovery and loading, and reactions lacking reverse transcriptase controlled for PCR contamination (-RT).

Figure 2.

Embryonic expression of *Panza* and *Edd*. Whole-mount in situ hybridization for *Panza* (A,A1,A2,C,E,E1,E2,G,I) and *Edd* (B,B1,B2,D,F,F1,F2,H,J) at stages 14 (A,A1,A2,B,B1,B2), 17 (C,D), 20 (E,E1,E2,F,F1,F2), 26 (G,H), and 33 (I,J). *Panza* expression is first detected at the neurula stage in the dorsal primitive gut (archenteron roof) extending from the oral evagination to the blastopore, with expression extending more laterally in the posterior gut. With elongation of the body axis, *Panza* extends along the entire anterior-posterior axis in the dorsal gut endoderm, narrower in the anterior gut and gradually broadening in the posterior gut. *Panza* is also expressed in the chordaneural hinge, extending into the floorplate of the posterior neural tube (arrowheads). *Edd* is expressed throughout the dorsal, lateral and ventral

endoderm, but not in the neural tube. Lateral views of cleared embryos with anterior left are shown. (A1–F2) Transverse sections (dorsal up) of stained embryos at the axial levels indicated by dotted lines in (A,B,E,F). fp; floor plate; n, notochord.

Figure 3.

Panza expression adjacent to the notochord. (A) Double staining for *Panza* (blue) by whole-mount in situ hybridization and notochord (brown) by immunohistochemistry using the Tor70 monoclonal antibody. *Panza* is expressed in the gut endoderm adjacent to the notochord and extends around the posterior tip of the notochord into the floorplate of the posterior neural tube. (A1–A4) Transverse sections of double-stained embryos (stage 33) at the axial levels indicated by dotted lines in (A). *Panza* is expressed in the dorsal endoderm of the foregut at the level of the eyes (A1), in the dorsal endoderm adjacent to the notochord in the anterior midgut (A2), and in the dorsal and lateral endoderm of the posterior midgut and hindgut (A3–A4). At the level of the hindgut, *Panza* expression is also detected in the floorplate of the posterior neural tube (A4). Higher magnification view of a section with the hypochord clearly visible (B). Higher magnification views of *Panza* and notochord in the posterior end at stage 20 (C), and in the elongating tailbud at stages 33 (D) and 36 (E), showing *Panza* expression ventral, posterior and dorsal to the notochord. Shown are lateral views of cleared embryos with anterior left (A,C–E) and transverse views with dorsal up (A1–A4,B). de, dorsal endoderm; e, eyes; fg, foregut; fp, floor plate; h, hypochord; hg, hindgut; mg, midgut; n, notochord; nt, neural tube; s, somite.

Figure 4.

Panza and *Edd* expression in the coiled gut and liver. In situ hybridization for *Panza* (A,A',C,C',E,E',G,G',I) and *Edd* (B,D,F,H,J) expression in guts removed from embryos at stages 40 (A,A',B), 41 (C,C',D), 42 (E,E',F), 44 (G,G',H), and 46 (I,J). Ventral (A,C,E,G) and dorsal (A',B,C',D,E',F,G',H) views with anterior left are shown. For some samples the coiled gut was straightened before in situ hybridization and is shown with anterior left (I,J). The brown material apparent in the coiled gut shown in (J) is gut contents present before sample preparation. Arrowheads indicate the position of the liver. gb, gallbladder; lv, liver; p, pancreas; s, stomach.

Expression of *Panza*, a *Xenopus* $\alpha 2$ -macroglobulin, in the dorsal endoderm adjacent to the notochord. A tailbud stage embryo (stage 33) was stained for *Panza* mRNA (blue) by whole-mount in situ hybridization and notochord (brown) by immunohistochemistry using the Tor70 monoclonal antibody. The embryo was cleared in benzyl benzoate-benzyl alcohol and photographed with transmitted illumination. A lateral view of the head and anterior trunk is shown.