Novel isoforms of synexin in *Xenopus laevis*: multiple tandem PGQM repeats distinguish mRNAs in specific adult tissues and embryonic stages

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Synexin (annexin VII) is a calcium-dependent, phospholipid-binding and membrane fusion protein in the annexin gene family, which forms calcium channels and may play a role in exocytotic secretion. We report here the cloning and characterization of five novel isoforms of cDNAs encoding *Xenopus* synexin from brain, oocyte and stage 24 cDNA libraries. The most prevalent *Xenopus* synexin has 1976 bp of cDNA sequence, which contains a 1539 bp open reading frame of 512 amino acids encoding a 54 kDa protein. This *Xenopus* protein is 6 kDa larger than the previously reported human and mouse synexins with which it shares approx. 73% identity in the C-terminal region and approx. 44% identity in the N-terminal region. Further studies with PCR revealed the molecular basis of the substantial divergence in the *Xenopus* synexin's N-terminal domain. The domain equivalent to the mammalian tissue-specific cassette exon occurs at a different position and is variable in size and

sequence. The most interesting observation relates to the occurrence of different forms of synexin due to the varying numbers of tandem PGQM repeats that are expressed differently in different adult tissues and embryonic stages. For these reasons we have labelled this set of unique isoforms annexin VIIb, referring to mammalian forms, which lack the PGQM tandem repeats, as annexin VIIa. In spite of these differences from annexin VIIa, the form of recombinant annexin VIIb with three PGQM repeats was found to be catalytically active. We interpret these results to indicate that the actual calcium and phospholipid binding sites are conserved in *Xenopus*, and that the variations observed between members of the synexin gene family in the regulatory domain clearly point towards the tissue- and stage-specific roles of individual members, possibly involving the exocytotic process.

INTRODUCTION

Synexin (annexin VII) is a calcium-dependent, phospholipid-binding and membrane fusion protein [1,2] in the annexin gene family [3,4], which forms calcium channels [5,6] and is thought to play a role in exocytotic secretion [7,8]. The potential role for synexin in the secretion process may be to serve as both a calcium sensor and as a fusion protein. For example, synexin has the requisite low affinity for calcium [9–12] and is localized, *in situ*, to secretory granule membranes in chromaffin cells [13]. Furthermore the rate of membrane fusion driven by synexin is approx. 4 μ s [8], which is appreciably faster than the 200 μ s estimated for synaptic transmission [14]. Synexin is also widely distributed throughout phylogeny, and human [3], mouse [15] and *Dictyostelium* [16,17] synexins have been cloned and sequenced.

In mammalian species, synexin is found in most tissues. In addition, an alternatively spliced form of synexin, using cassette exon 6 in the unique N-terminal domain, is expressed in skeletal and heart muscle and brain tissue in human [18], monkey [18], mouse [19] and cow [19]. The 22 amino acid sequence defined by cassette exon 6 in mammalian synexins seems to be a general mechanism for tissue-specific regulation of synexin expression in the four mammalian species studied. With minor variations it is highly conserved, and in monkey the expression of synexin with the cassette exon does not seem to be developmentally controlled [18]. We therefore hypothesized that the same regulating mechanism might be found in lower species such as *Xenopus laevis*.

To test this hypothesis, we cloned synexin from three *Xenopus* cDNA libraries and examined the nature of mRNA transcripts in the cassette exon region from different tissues and developmental stages. Remarkably we found that the mammalian regulating pattern was completely absent. Instead, a domain having weak homology to the cassette exon was constitutively expressed. However, in close juxtaposition to the location of the cassette exon we found varying numbers of tandem repeats of the four amino acid sequence Pro-Gly-Gln-Met (PGQM), suggestive of multiple forms within the regulatory region of the synexin molecule, occurring in a tissue- and stage-specific manner. Although equivalent roles might be served by these different types of domains, the capability of expressing multiple PGQM repeats during amphibian embryogenesis and development suggests a greater degree of subtle regulation in the simpler organism.

MATERIALS AND METHODS

Isolation of *Xenopus* synexin cDNA clones and DNA sequencing

Xenopus oocyte cDNA library in λ gt11 (a gift from Dr. Doug Melton, Harvard University, Cambridge, MA, U.S.A.) was screened under low stringency conditions by the standard methods [20] at 37 °C with a mouse synexin cDNA probe isolated in our laboratory [15]. The probe was labelled with [α -³²P]dCTP by nick translation (BRL). Out of 20 clones that

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1  TGGAGCTCCTGCAACCATCTGACAAGAATGTCGTACCAATACCGCTCAGGCTTCCCAAGGA
    M S Y Q Y P S G F P G 11
61  ATCCAGGCTATCCTGGAGGAGACCCATCATATCCACAGCAGCGCAACAGGCTTTCCT
    Y P G Y P G G D P S Y P P A A Q A F C P 31
121 GGGGACAATTCCTCCCTGCTGCTGAGGAGGCGCATTCACACAGCATCTGGTGGAGGG
    G G Q F P P A A G G G A F P P A S G G G 51
181 AATGCACCTCCTCCTGGCGGAGGGTATCCTCACGACAGGAGATACCCAGCTCCTGGTGGC
    N A P P P P G G G Y P H A G G Y P A P G G 71
241 TATCCTGGAGGCATGCCATCTTACCTGGAGCACCAGGCTTTGGAGCACCTGCTGGTGGC
    Y P G G M P S Y P G A P G F G A P A G G 91
301 CAAGGTATGGTGGCCCTCTGGTGCACCTGCTTATGGAGTCCCTGGGTATGGCGGGCT
    G G Y G P P G G A P A Y G V P G Y G G P 111
361 GGTTTTAAATGCTCCAGCTGGTGGATATGGGCTCCTAATGCAGGGGTTCGGGGTACCC
    G F N A P A G G Y G A P N A G G F G V P 131
421 CCAGCTGGTGGATATGGATCACCTGGAGGTGACACAGGCTATGGTGGTTTTCACACCT
    P A G G P G A P P G A P A Y G Y G G F S Q P 151
481 TCATCTCAGAGCTATGGAGCAGGAGGGCAGGACAAATGCCAGGACAAATGCCAGGACAA
    S S Q S Y G A G G P G Q M P G Q M P G Q 171
541 ATGCCAGGCCAAGGCCAAGTGATATCCAAGTGGCCGGCACCTGCACAACCTACGGCT
    M P G G A P P G A P A P A P A P A P A P 191
601 TATGCAGCAGGATGACAGGCACCTCAGGGCACAATTAAGCAGCACCTAATCTTGGC
    Y A A A M T A T Q G T I K A A P N F D A 211
661 CTATCTGACCTGAAAAGCTTAAGAAAGCAATGAAAGATTGAAACCGACGAGAAACCT
    L S D A E K L R K A M K G F G T D E K P 231
721 ATTGACGTTGTGCTAACCTTCCAATGACGAGACAAAAGATTGAGCGGCATTCAAG
    I D V V A A N R S N D Q R K I Q A A F K 251
781 ACCGCTTATGGCAAGATTTAATAAAGATCGAAGTCAGAAATTAAGTGGTAATGTGAA
    T A Y G K D L I K D L K S E L S G N V E 271
841 GAACCTATTATGCCCTTTTATCTCCACATATTATGATGCTGGAGCTTATATAT
    E L I I A L F M P S T Y Y D A W S L Y N 291
901 GCAATGAAGGAGCAGGAACACAGAGCGAGTACTCATTGAGATTCTATGCACAAGGACA
    A M K G A G T Q E R V L I E I L C T R T 311
961 AACAGTGAATCAGGAACATCGTTCCTGTACAAACAGAGTTTGGCAGGAAATTGAG
    N S E I R N I V A C Y K Q E F G R E I 331
1021 AAGGACATTCGATCAGATCTCTGCGGCAATTTGAGAGACTGCTGATATCTATCATGGCC
    K D I R S D T S G H F E R L L I S I M A 351
1081 AGGGGAATCTGTGATGAAAGCCAAATGTTAATCGACAGGCTGAGCAGGAGCCCA
    R G I V D G S Q N V N M Q O G Q D A Q 371
1141 CGCCTTTACCAAGCAGGGAAGGAAGCTTGAACAGACGAGTCTCCTTCAATTTGGTG
    R L Y Q A G E G K L G T D E S S F N L V 391
1201 TTAGCAAGTCGAAGTTTCCCCAGTTGAAAGCTGTGGCTGAAGCATATGCCAGGATATCC
    L A S R S F P Q L K A V A E A Y A R I S 411
1261 AAACGTGACCTGCTTAGTGTCATTGGCAGAGAATTTTCTGGATATATAGAAGATGGCCTG
    K R D L L S V I G R E F S G Y I E D G L 431
1321 AAAGCAGTTTTCAGTCCGCTATCAACCGTCTCTTTCTTGTGATAGACTTTGCCGC
    K A V L T G C A I N R P L F R D R A C L T C 451
1381 TCTATGAAGGAGCGGACCTGATGATTCACCGCTGATAAGGATTATGTACCCAGAACG
    S M K G A G T D D S T L I R I I V T R S 471
1441 GAGATTGATCTGTGCGAGATCAACAGGCTTATGTACAGATGTACAGAAATCTCTTCT
    E I D L V Q I K Q A Y V Q M Y Q K S L S 491
1501 GCAGCAATCTCGAGGATACCTCCGGGGCTACAAGCGCATGCTGTTAGCAATTTCTGGC
    A A I S S D T S G A Y K R M L L A I S G 511
1561 CACTAGGATCTATCTGTGAATGAAATATATGTCAGATAAACTATATGTCCATATCTA
    H *
1621 TATATAGATCGAAATGATATCTGCAACTCATTATAGGAAATTTGATCATGTCTATTATA
    CCATAAACGTAATGTTGTGTCATGTTAGATTTTGGCATATTTTACACACATGCCGTA
1741 TATATAAATATAAGCTATAGAGCTGGCTTTCACATTCAGCAAAATGTGGCATAATA
1801 TACACCCAGTGCTTTTGTACTCGAGAAATCTAAATCAGTGGAGAAAGAAAAACCTGAT
1861 GTGACCAAGTTTCAGGAAGTTAAGGAATTTCTCTCTTTGCAATTAATGTCATTTTCATG
1921 ACGGTTTGTTCAGTAAAAAACCTCAAGCTCAAAAAAAGAAAAAAGAAAAA

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Figure 1 Nucleotide and deduced amino acid sequences of full-length *Xenopus* synexin clones

Shown are 27 bases of 5'-non-coding sequence followed by the translation initiation site and the coding sequence of clone XSB3. Numbers at the left indicate the position of the nucleotide and at the right the position of the amino acid. The termination codon (starred) is followed by 410 bases of 3'-non-coding sequence. The oligonucleotides described in the Materials and methods section are underlined, as is the poly(A) signal (AATAAT) in the 3'-untranslated region.

hybridized to the probe, 10 were plaque purified, excised and subcloned into the pGX 2627 vector for double-stranded plasmid DNA sequencing by the dideoxynucleotide chain termination method with Sequenase T4 DNA polymerase in accordance with the manufacturer's instructions (U.S. Biochemical Corp.). Oligonucleotides to internal sequences were synthesized with an

Applied Biosystems model 380B DNA synthesizer. Because none was a full-length clone, the longest of the cDNAs was used as a probe to screen a *Xenopus* brain library (a gift from Dr. Igor Dawid). The positive clones were rehybridized with the oligonucleotide 5'-TCCCTGGGTATGGCGGGCCTGGTTT-3' (XSRP8; 341–365, Figure 1) derived from the upstream region of the longest clone XO1 (Figure 2). Two clones were found that hybridized with both the oligonucleotide and the cDNA probe. These were plaque purified, subcloned and sequenced. The upstream region still lacked the translation initiation codon, and we therefore performed PCR amplification to obtain the remainder of the N-terminal domain. The template was derived from the cDNA libraries of *Xenopus* brain, oocyte and the stage 24 embryo. The first set of primers used were the λ gt11 forward primer 5'-CUACUACUACUAGGTGGCGACGACTCCTGGAGCC-3' as sense primer and the specific *Xenopus* synexin sequence 5'-CAUCAUCAUATTTTCAGCATCAGATAGGGCGT-3' (XSUS1; 656–677, Figure 1) as antisense primer. The second set of primers used were both from the *Xenopus* synexin sequence. The sense oligonucleotide sequence 5'-CUACUACUACUAGGTTTTCACAACCTTCATCTC-3' (XSUS1; 465–486, Figure 1) is from upstream of the cassette region of the mammalian synexin, and the antisense primer 5'-CAUCAUCAUAGTGGCCAGAAATTGCTAACAG-3' (XSUS2; 1541–1563, Figure 1) is near the stop codon. All of the oligonucleotides had U in their anchor sequences of the first 12 base pairs (CUACUACUACUA) to facilitate cloning of the PCR products directly into the plasmids. The PCR products were subcloned into the UDG-cloning vector in accordance with the manufacturer's instructions (Gibco-BRL) and sequenced. Sequence data were analysed with the PCgene software (Intelligenetics Inc.).

RNA isolation

Total RNA was isolated from *Xenopus* oocytes and from adult liver, lung, muscle, motor neurons, kidney, skin, small intestine and brain by using RNazol (Cinna-Biotech).

Reverse transcriptase/PCR (RT/PCR)

RT/PCR was performed as described by the supplier of the enzyme (Perkin-Elmer Cetus Instruments). Briefly, 4 μ g of total RNA was transcribed into single-stranded DNA by using Moloney murine leukaemia virus reverse transcriptase (Gibco-BRL) and 10 pmol of synexin-specific antisense primer. In the negative control samples, the template DNA was omitted from the PCR reactions and subjected to the same conditions as above. The PCR products were then analysed electrophoretically on a 1.0% (w/v) agarose gel containing 50% additive (Integrated Systems) and revealed by ethidium bromide staining.

Construction of full-length *Xenopus* synexin (FLX)

Because the full-length sequence was obtained by a combination of two methods, a construct was designed to test whether the open reading frame would indeed encode a catalytically active synexin. The sequence to be tested was defined by the translation initiation start site contained in the clone XSB derived from the PCR amplification, and the stop site present in another clone XB2 derived from screening the library. The KPN 1 restriction site present at the 3' end of clone XSB and at the 5' end of clone

XB2 was chosen as a common site for restriction. We synthesized two oligonucleotides, an antisense primer 5'-CAGCTGGGGG-TACCCCGAA-3' (XSAKPN; 409–426, Figure 1) from the former, and a sense primer 5'-TTCGGGGTACCCCGAGCTG-3' (XSKPN; 409–426, Figure 1) from the latter containing the *Kpn*I restriction site. Two other oligonucleotides were designed to contain *Sal*I and *Bam*HI restriction sites for cloning into the pET 12 plasmid. The sense primer 5'-CTGAGGTCGACATGTCGTACCAATACCCGTCA-3' (XSSAL; 28–48, Figure 1) was made from the 5' end of clone XSB with the *Sal*I restriction site. The antisense primer 5'-CTCAGGGATCCCTAGTGCCAGAAATTGCTAACAG-3' (XASBAM; 1541–1566, Figure 1) was made from the 3' end of clone XB2 with the *Bam*HI restriction site. PCR amplification was carried out with clone XSB as a template and XSSAL and XSAKPN as primers, and the product obtained was restricted with *Sal*I and *Kpn*I. With clone XB2 as a template and XSKPN and XASBAM as primers, the PCR amplification was carried out and the product was digested with *Kpn*I and *Bam*HI. The two products were gel purified with Gene Clean (Bio 101) and ligated to a pET 12 plasmid that had previously been restricted with *Sal*I and *Bam*HI, to produce the recombinant *Xenopus* synexin expression vector FLX.

Expression of recombinant *Xenopus* synexin

The DH5 α cells, containing either the pET 12 parent vector or the FLX expression vector, were grown in Luria broth with 100 μ g/ml ampicillin, induced with 10 mM isopropyl β -D-thiogalactoside overnight, and lysed with 40 μ g/ml lysozyme in the presence of 25 mM Tris/HCl, pH 7.5, containing 10 mM EGTA, 5 mM MgCl₂, DNase 1 and an antiprotease cocktail. The cocktail contained 250 μ M PMSF, 20 μ M leupeptin, 5 μ M pepstatin and 1 mM benzamidine. After three repetitions of sonication and freeze-thawing, aliquots of total cell lysates were resolved by SDS/PAGE and the proteins transferred to nitrocellulose membrane. A major Coomassie Blue-stained band with an apparent M_r of approx. 54 was detected in extracts of cells containing the FLX expression vector but not in cells that contained the pET 12 parent plasmid. This band was also labelled by a Western blot analysis using a polyclonal antibody against a human synexin peptide sequence within the second repeat. The 54 kDa recombinant protein was purified from the DH5 α cell extract by double precipitation with ammonium sulphate, gel filtration on a ACA54 column and reversible calcium-dependent binding to PS liposomes [21].

Chromaffin granule aggregation assay

Aggregation was assayed by measuring the change in turbidity (D_{540}) of the chromaffin granule suspension induced by the recombinant *Xenopus* synexin and calcium as previously described [1]. Briefly, the assay was performed at room temperature in a 1 ml quartz cuvette containing the chromaffin granule suspension in 0.3 M sucrose, 40 mM histidine buffer, pH 6.0, 1 mM CaCl₂ and variable amounts of recombinant synexin from *Xenopus* and human.

RESULTS

Isolation of cDNA clones

Using mouse synexin cDNA as the probe, we isolated and sequenced ten hybridizing clones from a *Xenopus* oocyte cDNA library. The longest partial *Xenopus* synexin cDNA, lacking 196 bp from the translation initiation codon, was then used to screen a *Xenopus* brain cDNA library. Two clones were isolated that hybridized to the 5' probe. Further analysis of these clones

revealed that they still lacked 100 bp in the putative N-terminal region. However, by the method of PCR amplification of λ DNA derived from *Xenopus* oocyte, brain and stage 24 cDNA libraries resulted in defining the full-length sequence. In addition, subcloning and sequencing of several of these fragments, as described in the Materials and methods section, led to the identification of five different *Xenopus* synexins (Figure 2). The sequence of XSB3, the predominant species present in all three libraries, is shown in Figure 1. This cDNA is 1976 bp long, with 27 nucleotides in the 5'-untranslated region, an open reading frame of 1536 bases and the remainder in the 3' untranslated region. The polyadenylation signal is at position 1902 with a poly(A) tail of 22 bp.

To illustrate the relationships between the synexins we have aligned the protein coding sequences of three full-length *Xenopus* clones, which have 95% homology between themselves, with the mammalian and *Dictyostelium* synexin sequences (Figure 3). We noted that whereas human and mouse synexins are 93% identical, *Xenopus* synexin is 67% identical with human synexin and 68% identical with mouse synexin. By contrast, *Xenopus* synexin had only 40% identity with *Dictyostelium* synexin and 46% identity with annexin XI (results not shown). Most of the significant differences between the synexins are concentrated in the N-terminal domain, including residues 37–146 (human numbering). When comparing *Xenopus* with mammalian sequences it is evident that *Xenopus* synexin is 46–55 residues longer than mammalian synexin, and that the larger size is due to the insertions at positions 37, 76, 85 and 124. The *Xenopus* insertion at position 37 has either seven residues in clones XSB3 and XSD6 or 14 residues in clone XSC4; at position 76 there are 12 residues; at position 85 24 residues; and at position 124 there are varying numbers of tandem PGQM repeats. The clone XSB3 has three tandem PGQM repeats, XSC4 has four and XSD6 has six. The segment containing the tandem PGQM repeats in *Xenopus* is not present in mammalian synexins. Conversely, the mammalian cassette exon 6 at position 146, which is expressed only in heart, skeletal muscle and brain, is constitutively present in all the *Xenopus* synexins, albeit with weak homology. Finally, there were several conservative substitutions in the *Xenopus* synexin's conserved C-terminal domain, and the putative sites for phosphorylation, calcium and phospholipid binding in this domain are all conserved in *Xenopus* synexin. The dendrogram in Figure 4 clearly shows that the *Xenopus* synexin is most homologous with human synexin and is least homologous with annexin 10.

Multiple transcripts of synexin mRNAs in adult tissues

The differences that exist in the PGQM repeat region of these different *Xenopus* synexins raised the possibility of multiple transcripts with similar sequences. To test for the possible expression of mRNA variants in different tissues, two oligonucleotides flanking the PGQM repeat region were selected. Figure 5 shows the four products obtained by PCR amplification of total RNA from different tissues. To verify the identity of these PCR products they were all gel purified with Gene Clean and were subcloned into UDG cloning vector. Each band was sequenced with SP6 and T7 promotor as sequencing primers. We found that only heart, brain, motor neurons and muscle have band 1 because the sequence corresponded to the form with two PGQM tandem repeats. Band 2, corresponding to the form with three PGQM tandem repeats, is present in all the tissues. Band 3, with four PGQM tandem repeats, is present in all the tested tissues, although smaller amounts are present in brain and motor neurons. Band 4, when analysed, had a variable sequence with no

Figure 2 Diagrammatic representation of *Xenopus* synexin cDNAs

(A) Isolation of *Xenopus* synexin clones by the plaque hybridization technique. The solid bar represents the N-terminal sequence and the open box represents the four repeats. The term XO indicates that the cDNA library is from *Xenopus* oocyte. In the first screening, the term XO without numbers denotes the composite of 10 clones, grouped into four categories according to their size. In the second screening, clones XB2 and XB16 were obtained from an XB (*Xenopus* brain) cDNA library by hybridization with a 5'-oligonucleotide from the XO clones. **(B)** Isolation of *Xenopus* synexin clones by PCR amplification using λ DNA template made from XO, XB and X.St 24 cDNA libraries. **(a)** A λ gt11 forward primer and XSUS1, an antisense primer, were used to yield four clones XSA, XSB, XSC and XSD (identified on the left). The symbols on the right-hand side denote the library of origin. Inverted solid triangles represent sequence variations (see Figure 3). The short vertical lines represent multiples of the (PGQM) repeat. The (-) represents a substitution in the PGQM sequence (see Figure 7). With XSUS1 as sense primer and XSUS2 as antisense primer and the DNA template from different libraries, PCR amplification yielded six sets of clones. These are identified by the labels at the left in the lower set. The numbers denote the number of PGQM tandem repeats in each clone. The libraries from which each were obtained are shown on the right-hand side of the lower set. The superscripts, asterisks and hat symbols in XS3' and XS6' represent modifications noted in Figure 7. **(b)** Summary of the composite sequences obtained by plaque hybridization and PCR amplification techniques. The lower clone XSQ3 was derived separately by plaque hybridization.

PGQM repeat motif. Furthermore it did not appear in brain and motor neurons.

Multiple forms of synexin in different embryonic stages

Differential synexin expression during embryonic development was analysed in terms of the tandem PGQM repeat content by RT/PCR (see Figure 6). Each band was excised, gel purified using Gene Clean, subcloned into the UDG cloning vector and sequenced with SP6 and T7 promoters as primers. At stage 2 (two-cell stage), there are three bands corresponding to two (XS2), four (XS4) and five (XS5) PGQM tandem repeats. The mRNA transcript fragment with three PGQM tandem repeats is expressed well at stage 10 (gastrula stage), whereas it starts to decline at stages 16/17 (late neurula stage) and 37/38 (tadpole stage). Bands corresponding to four and five tandem PGQM repeats are seen at all stages of development. XS6 with six

tandem PGQM repeats is seen from stage 10/11. These results suggest that the expression of the *Xenopus* synexin gene is developmentally controlled, and that the number of PGQM tandem repeats in the unique N-terminal domain is important in this process.

Summary of *Xenopus* synexin mRNA variants

A set of sequences of mRNA variants occurring in the N-terminal domain can be compiled from library screening, PCR amplification of mRNA from different tissues and different developmental stages. As shown in Figure 7, XS6, XS6' and XS6'' have six tandem PGQM repeats. In XS6' Val is replaced by Cys at position 30. In XS6'', the XS6' mutation is present, and in addition Met is replaced by a Val at position 12. Whereas XS5, XS4 and XS3 have five, four and three PGQM tandem repeats respectively, XS3' has, in addition, replacement of Ala and Pro

Figure 3 Sequence comparison of synexins from different species

The deduced amino acid sequences of *Xenopus* synexins XSD6, XSB3 and XSC4 are compared with the amino acid sequences for human (HS) [3], mouse (MS) [15] and *Dictyostellium* (DS) [16,17] synexins. The multiple alignment program of PCgene was used. An asterisk indicates that the residue is perfectly conserved. A dot indicates that it is homologically conserved. A dash indicates a space introduced to optimize the alignment. The four conserved C-terminal calcium/phospholipid binding motifs are underlined. The positions of exon boundaries in the human [25] and mouse [19] synexin genes are indicated by numbered arrows above the sequence. ♦, Amino acid residues in the human annexin V crystal structure that were determined as being involved in calcium ion co-ordination. Unnumbered arrows (↓) indicate amino acid residues that are hypothesized to be involved in phospholipid-dependent calcium ion co-ordination. The amino acids shown in solid boxes represent the sets of unique residues inserted into *Xenopus* or human and mouse synexins. The dotted box indicates the phosphorylation site for various kinases in other annexins.

for Val and Tyr at positions 28 and 29 respectively. Between themselves, the mRNA variants in *Xenopus* have 55% identity. Yet when these sequences were compared in the same region with

Figure 4 Evolutionary tree based on sequence divergence in the highly conserved C-terminal domain of annexins

The relatedness tree was constructed with the multiple alignment program of PCgene, which plots a dendrogram showing the clustering relationships used to determine the order of the pairwise alignment that together create the final multiple alignment. Distance along the vertical axis is proportional to the difference between sequences; distance along the horizontal axis has no significance. See [4] for details on each annexin (Anx) gene. Dros., *Drosophila*.

Figure 5 Analysis of the mRNA variant fragments in the PGQM repeat region of *Xenopus* synexin gene by the RT/PCR method

The first-strand cDNA, prepared from the different tissues indicated, was subjected to PCR with primers XSUS1 and XSUAS1 (see Figure 1) as described in the Materials and methods section. Amplified products were separated on 1% (w/v) agarose gel with 50% additive (Integrated Systems) and stained with ethidium bromide. The numbered bands contain different tandem PGQM repeats: (1) XS2; (2) XS3; (3) XS4. Band 4, migrating most slowly, did not contain *Xenopus* synexin sequences as determined by sequence analysis. These amplification products were not visible in the negative control reactions where the RNA was not reverse transcribed (results not shown). Each RNA sample was subjected to RT/PCR analysis four times to verify the amplification pattern shown here. Abbreviations used: H, heart; MN, motor neurons; B, brain; Lg, lung; L, liver; M, muscle; O, oocyte; K, kidney; S, skin.

human, mouse and bovine, there is only 14% identity. Nevertheless the rest of the *Xenopus* synexin sequence is quite homologous with mammalian forms.

Bacterial expression of active recombinant *Xenopus* synexin

The *Xenopus* synexin cDNA was subcloned into the pET 12 vector and the protein was expressed after induction with

Figure 6 Developmental expression of PGQM repeat region in *Xenopus* determined by the RT/PCR method

An RNA sample from different stages of development (stages 2, 10, 16/17 and 37/38) and from a cell line (XTC) derived from *Xenopus* metamorphosing tadpole was subjected to RT/PCR and analysed on 1% (w/v) agarose gel as described in Figure 5.

isopropyl β -D-thiogalactoside. After purification as described in the Materials and methods section, the immunoreactive *Xenopus* recombinant protein was found to have a molecular mass of 54 kDa, as predicted by the cDNA sequence. This is 6 kDa more than human, mouse or bovine synexins. With this purified recombinant protein we next tested for its activity in the chromaffin granule aggregation assay. As shown in Figure 8, the activity increased as a function of increasing concentrations of protein in the presence of calcium. These data thus indicate that the recombinant *Xenopus* synexin is able to function in granule aggregation assays exactly like its human and mouse counterparts.

Figure 8 Chromaffin granule aggregation assay of *Xenopus* and human recombinants

The aggregation assay was carried out as described in the Materials and methods section. The change in turbidity was measured at 540 nm at different indicated concentrations (μg/ml) of the respective recombinant protein in the presence of 1 mM calcium. Data are means $\pm$ S.D. ($n = 3$).

DISCUSSION

In mammalian synexins, tissue-specific expression depends on the alternative splicing of the 66 bp cassette exon 6 and synthesis of synexin with a 22 residue insert at position 146 in the unique N-terminal domain [18]. This sequence INTDSFSSYPVFSPPVSLDYSS introduces charged residues into an otherwise hydrophobic domain, and these charged sites are conserved across the four mammalian species studied. We therefore anticipated that such a conserved regulating domain might also be evolutionarily conserved in lower vertebrates such as *Xenopus laevis*. However, as shown in the present paper, an entirely different result was

Figure 7 Comparison of the amino acid sequences between different mRNA variants of synexin from different tissues and at different stages with the cassette exon region (boxed) of human, mouse and bovine synexins within exon 5 and 6

XS denotes that the clone is *Xenopus* synexin and the different numbers indicate the number of tandem PGQM repeats. The superscripts indicate individual variations. An asterisk indicates complete identity and a dot indicates the homology between all residues. #, A site of identity or the homology where a complete set of residues is not present. The sequences are aligned with the inclusion of gaps (indicated by dashes) to emphasize the similarities between the protein sequences. The numbered arrows indicate splice junctions. HSCP, human synexin; MSCP, mouse synexin; BSCP, bovine synexin.

obtained when we examined the *Xenopus* synexins. First, instead of two forms of synexin, at least five forms were detectable based on distinct cDNAs. Secondly, the alternatively edited cassette exon 6 was constitutively present in all the *Xenopus* synexins studied, albeit with modestly reduced homology to the mammalian sequences. For example, the three acidic residues in the mammalian cassette exon domain were replaced with two neutral residues and one hydrophobic residue in the *Xenopus* sequence. Lastly, in place of the variably expressed mammalian cassette exon 6 we found instead that the tissue-specific domain in *Xenopus* synexin was a closely juxtaposed upstream sequence consisting of multiple tandem repeats of PGQM. The PGQM tandem repeat thus seems to be a functional alternative to the mammalian cassette exon domain. These results thus also point to a regulatory aspect of gene expression that has not previously been reported for synexin during embryonic development. A computer search against the PGQM repeat in the protein database revealed its existence in the *gag* region of the HIV gene, the 70 kDa heat shock protein, and the collagen $\alpha 1$ (VIII) precursor. However, no relevant functional significance has been attached to it in any of these cases.

Finally, the fact that the bacterially expressed recombinant *Xenopus* synexin is able to aggregate chromaffin granules in the cell-free reconstitution experiment lends clear support to the concept that synexin could indeed support exocytotic secretion in the *Xenopus* oocyte system. The oocyte system supports calcium-dependent secretion of transmitters such as glutamate [22] and acetylcholine [23] when the appropriate biosynthetic enzymes are expressed recombinantly. Furthermore it has been possible to implant chromaffin granules and chromaffin granule ghosts into intact oocytes, and to detect calcium-stimulated exocytosis of granule contents and appearance of granule membrane dopamine mono-oxygenase (D β H) on the external surface of the oocyte [24]. The intriguing conjunction of chromaffin granules, with which we have had substantial experience [21], and the oocyte indicated to us that with the results now in hand *Xenopus* might provide an attractive system to test the synexin hypothesis for exocytosis. However, the complicating concern is that the oocyte contains all the PGQM variations. It is this very diversity of the different *Xenopus* synexins that could profoundly complicate the possibility of using *Xenopus* cells to assess this specific function. Indeed, these results could be further evidence for the supposition that the variant synexins could be important for developmental processes beyond simple exocytosis. We therefore expect that comparison of different isoforms of synexin with respect to subcellular localization, calcium-dependent aggregation of

chromaffin granules, membrane fusion and channel properties will shed light on the structural basis of differential synexin function during development.

We conclude that in the evolutionarily lower vertebrate *Xenopus laevis*, expression and function of synexin depends on differential expression of a unique, tandem PGQM repeat in the N-terminal domain of the protein. In contrast, in higher vertebrate mammalian species, synexin lacks the tandem PGQM repeat altogether, and instead uses an alternately spliced 22 amino acid cassette exon.

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