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Original Article

Exploration of the extracellular space by a large-scale secretion screen in the early *Xenopus* embryo

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ABSTRACT Secreted proteins play a crucial role in intercellular communication during embryogenesis and in the adult. We recently described a novel method, designated as secretion cloning, that allows identifying extracellular proteins exclusively based on their ability to be secreted by transfected cells. In this paper, we present the results of a large-scale screening of more than 90,000 clones from three cDNA expression libraries constructed from early *Xenopus* embryos. Of 170 sequenced clones, 65 appeared to encode secreted proteins; 26 clones (40%) were identical to previously known *Xenopus* genes, 25 clones (38%) were homologous to other genes identified in various organisms and 14 clones (22%) were novel. Apart from these *bona fide* secreted proteins, we also isolated lysosomal or other secretory pathway proteins and some cytoplasmic proteins commonly found in body fluids. Among the novel secreted proteins were two putative growth factors of the Granulin family, termed xGra1 and xGra2; they are structurally similar to EGF and TGFα and show a spotted expression pattern in the epidermis. Another secreted protein, designated xSOUL, belongs to the family of heme-binding proteins and exhibits distinct expression in the early brain. A third protein, termed Xystatin, is related to cysteine proteinase inhibitors. Our results indicate that secretion cloning is an effective and generally useful tool for the unbiased isolation of secreted proteins.

KEY WORDS: *secretion cloning, maternal, gastrula, Spemann-Mangold organizer, lysosomal*

Introduction

A major challenge in developmental biology is to explore the molecular mechanisms that underly cell-cell interactions. The importance of intercellular signaling has long been appreciated in inductive processes, in which one tissue emits signals that change the fate of its neighbors. A classic example is the Spemann-Mangold organizer, a region of the amphibian gastrula, which is a source of signals that induce the central nervous system and pattern the early embryo (Spemann and Mangold, 1924). More recently, key roles for extracellular signaling have been recognized in important biological processes such as growth, differentiation and cancer progression. Studies in *Xenopus* have provided much insight into our understanding of cell-cell signaling. Secreted proteins and their cell surface receptors regulate many developmental processes. For example, members of the bone morphogenetic protein (BMP), Activin, Hedgehog and Wnt family, along with fibroblast growth factor (FGF) and Notch proteins, are critical for patterning and morphogenesis. The normal function of tissue requires elaborate mechanisms for the cells to send signals and exchange information. Extracellular proteins may bind to the ligands or receptors and serve as positive or negative modulators. These are frequently subject to proteolytic cleavage by enzymes, which in turn may be blocked by protease inhibitors. Thus, different layers of regulation exist that modulate growth factor signaling in the extracellular space (reviewed in De Robertis and Kuroda, 2004). Because of the important developmental roles of secreted proteins, particular efforts have been made to identify genes encoding such proteins. A common feature of extracellular proteins that can be used for their isolation is the presence of a

Abbreviations used in this paper: BLAST, basic local alignment search tool; BMP, bone morphogenetic protein; EGF, epidermal growth factor; ER, endoplasmic reticulum; EST, expressed sequence tag; FGF, fibroblast growth factor; IGF, insulin-like growth factor; kD, kilo Dalton; LiCl, lithium chloride; NCBI, national center for biotechnology information; S, sulfur; SDS-PAGE, sodium dodecylsulphate polyacrylamide gel electrophoresis; TGF, transforming growth factor; UV, ultraviolet light; xGra, *Xenopus* Granulin.

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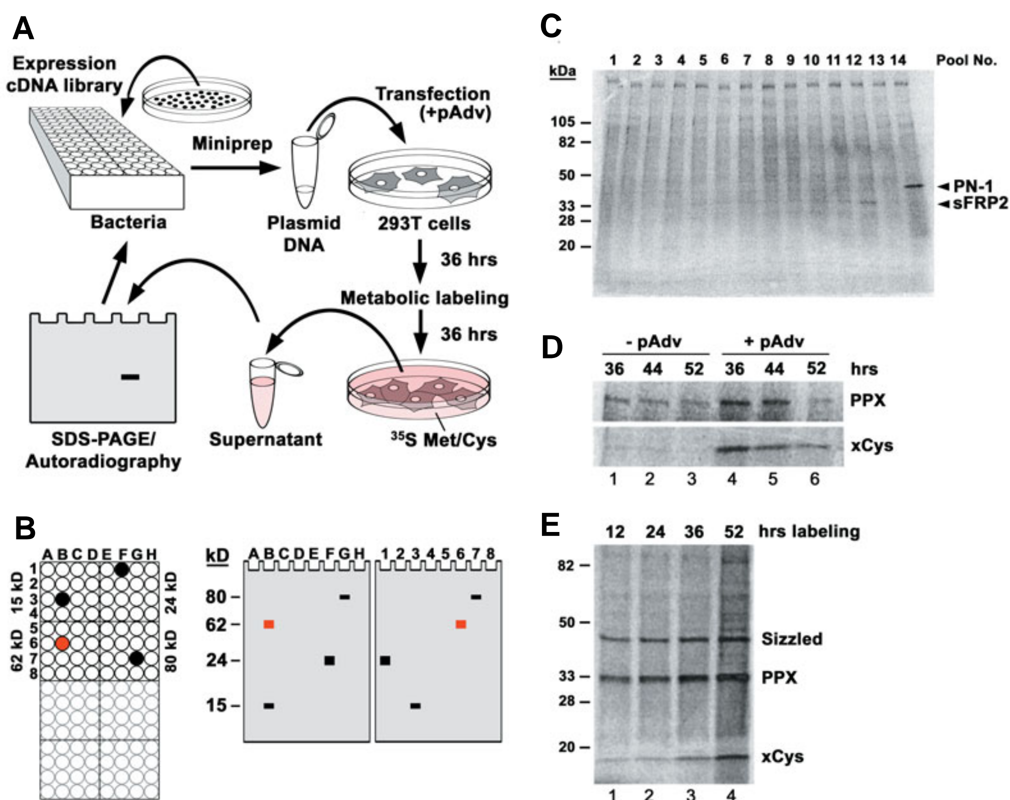


Fig. 1. Secretion Cloning: a novel approach to isolate secreted proteins. (A) *Overview of the method. Bacterial colonies from expression cDNA libraries are picked from agar plates and individually grown in 96 well blocks. Culture media from 16 clones are pooled and plasmid DNA prepared by miniprep. Human embryonic kidney 293T cells are co-transfected with the cDNA pools and pAdvantage (pAdv) DNA. After 36 hours, the transfected cells are labeled with ^{35}S-Methionine/^{35}S-Cysteine and incubated for another 36 hours. Proteins in the cell supernatant are separated by SDS-PAGE and detected by autoradiography. Once a candidate pool is identified, the procedure is repeated using subpools to individualize the positive cDNA clone.* **(B)** *Method of sib-selection. Left panel, bacteria from selected pools were regrown in a 96 well block and the 16 clones of each pool arrayed in 4x4 wells. The molecular weight of the candidate protein in each pool is indicated on the side. Note that pools grouped next to each other should differ in their molecular weight. Right panel, autoradiogram of protein gels. Each lane is loaded with the supernatant of cells transfected with a subpool of eight cDNA clones, prepared from one column (letter) or one row (number) of the 96 well block. Note that a positive clone is identified, when the bands in two lanes have the same molecular weight as the original pool.* **(C)** *Example of an SDS-PAGE of radioactive supernatants of 293T cells transfected with pools of 16 cDNA clones from a* Xenopus *gastrula library. Note in lanes 12 and 14 bands of 34 and 45 kD, respectively, which led to the initial identification of secreted Frizzled-related Protein-2 (sFRP2) and Protease inhibitor Nexin-1 (PN-1) in* Xenopus. **(D)** *Optimization of transfection conditions; cDNA clones encoding Protein Phosphatase X (PPX) or* Xenopus *Cystatin (xCys) were transfected alone (lanes 1-3) or with 10% pAdvantage DNA (pAdv, lanes 4-6). The time between transfection and the addition of ^{35}S-methionine/^{35}S-cysteine is indicated in hours. Note that the protein yield is highest in the presence of pAdvantage and when metabolic labeling was started 36 hours after transfection.* **(E)** *Detection of Sizzled, PPX and xCys protein in the medium of transfected cells after varying lengths of radioactive labeling. Note that conditioning for 36 hours yields strong signals of the desired proteins, while longer incubation increases non-specific background.*

signal peptide, i.e. a cleavable sequence of 15-30 mainly hydrophobic amino acids at the amino-terminus, which confers passage of the protein into the secretory pathway (Bendtsen *et al.*, 2004). Although there is little sequence conservation among signal peptides, their uniformity of function can be exploited in a gene isolation strategy termed signal sequence trap. cDNAs encoding signal peptides can redirect the expression of an export-defective receptor to the surface of mammalian cultured cells (Tashiro *et al.*, 1999). Alternatively, cDNAs encoding extracellular proteins can be selected based upon their ability to provide a functional signal peptide to a truncated enzyme in yeast (Klein *et al.*, 1996). In mice, reporter gene insertions into genes encoding secreted proteins („secretory traps“) have been identified in embryonic stem cells based on membrane insertion of β-Gal fusions with „trapped“ signal peptides (Skarnes *et al.*, 1995). However, these approaches are not exclusively directed against secreted proteins, but they also target transmembrane proteins or proteins localized to the endoplasmic reticulum, Golgi apparatus and lysosomes. Importantly, the isolated cDNA clones are only partial and additional effort needs to be subsequently made in each case to isolate full-length cDNAs for functional studies. We have reported a novel method, termed secretion cloning, to directly isolate full-length cDNA clones encoding secreted proteins (Pera and De Robertis, 2000). Secreted proteins were identified by monitoring the supernatant of human 293T cells transfected with small pools of cDNA clones from *Xenopus* expression cDNA libraries. Following metabolic labeling with ^{35}S-methionine and ^{35}S-cysteine, proteins released into the culture medium were separated by gel electrophoresis and detected by autoradiography. This unbiased method allowed the isolation of secreted proteins as full-length cDNA clones that could be directly used for functional characterization in mRNA microinjection experiments. Using this method, we have so far reported on the isolation of four soluble Wnt antagonists of the secreted Frizzled-related protein family, namely Frzb-1, Sizzled, sFRP-2 and Crescent, which exhibit distinct activities in early head development (Pera and De Robertis, 2000). We also isolated the Nodal antagonist Antivin and this construct contributed to the realization that neural induction occurs in the absence of mesoderm (Wessely *et al.*, 2001). Another secreted protein, termed IGFBP5, led us to describe a new role for insulin-like growth factor (IGF) signaling in head and neural induction (Pera *et al.*, 2001). In addition, we

presented three novel secreted proteins, including Isthmin as a new member of the FGF synexpression group (Pera *et al.*, 2002) and two members of the glutamate carboxypeptidase family, designated as Darmin and Darmin-related (Pera *et al.*, 2003). Despite these individual reports on some isolates, this comprehensive screen has never been published as a whole. Here we summarize our results and provide a complete overview of screening 91,376 clones from three different *Xenopus* cDNA libraries. We present 87 new ESTs and three full-length cDNA sequences of secreted proteins and other components of the secretory pathway. In addition, some cytoplasmic and nuclear proteins were retrieved in our screen of ^{35}S-labelled proteins. Furthermore, we present here the expression patterns of six selected genes encoding secretory proteins. Our results demonstrate that secretion cloning is well suited to explore the molecular composition of the extracellular space and to identify novel secreted proteins.

Results

Strategy of secretion cloning

To explore the molecular composition of the extracellular space, we performed an unbiased screen for proteins secreted in the early *Xenopus* embryo (Pera and De Robertis, 2000). We developed a novel method that allows one to directly isolate full-length cDNA clones encoding secreted proteins (Fig. 1; for details, see Materials and Methods). Human 293T cells were transfected with small pools of cDNA clones from *Xenopus* expression libraries and newly synthesized proteins were radioactively labeled with ^{35}S-Methionine and ^{35}S-Cysteine (Fig. 1A). Proteins secreted into the supernatant were detected by SDS-PAGE and autoradiography. Positive clones were individualized in a second step using sib selection (Fig. 1B) and partially sequenced from the 5' end to reveal their identity. One important criterion in the course of the screen was that many secreted proteins undergo glycosylation, while passing the ER and the Golgi apparatus. Hence secreted proteins generate a diffuse smear (rather than distinct band) when separated by gel electrophoresis (Fig. 1C). In order to improve the secretion of proteins, we transfected cDNA clones previously isolated by secretion cloning and examined different culture conditions. The pAdvantage plasmid (Promega) encodes the adenoviral Virus Associated I (VAI) RNA, which increases transient protein expression in various cell lines probably by stimulating translation initiation; VAI RNA is thought to relieve suppression of the translation initiation factor eIF-2 by the double-strand RNA-activated inhibitor (DAI) that is activated by transfection. Co-transfection of cDNA with pAdvantage into 293T cells significantly enhanced the protein yield as shown in Figure 1D (compare lanes 4-6 with 1-3). The strongest signal was obtained, when the transfected cells were incubated for 36 hours before the onset of metabolic labeling. Longer incubation reduced protein biosynthesis, perhaps because cells reached confluence and exited the cell cycle. We further investigated the optimal length of metabolic labeling (Fig. 1E). After addition of ^{35}S-Methionine and ^{35}S-Cysteine, proteins synthesized from the transfected cDNA were best detected after 36 hours (Fig. 1E; lanes 1-3). Harvesting the supernatant at a later time point was disadvantageous, since background accumulated due to the release of non-specific proteins from lysing cells (Fig. 1E; lane 4).

Spectrum of secreted proteins

In total, we sceened 91,376 clones from three different expression cDNA libraries (Fig. 2A). One library was prepared from 32-cell stage embryos representing maternally expressed mRNAs. The source of the two other libraries were gastrula embryos that have been partially ventralized by UV light exposure or dorsalized by lithium chloride (LiCl) treatment, respectively. We isolated 170 cDNA clones that showed radioactively labeled bands in several independent transfection experiments, partially sequenced them from the 5' end and analyzed them by BLAST to

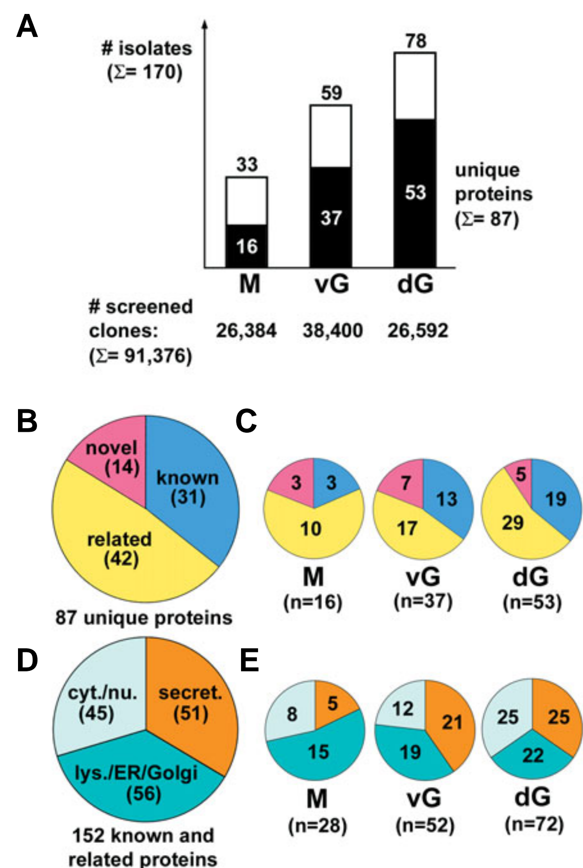


Fig. 2. Overview of the proteins isolated by secretion cloning. (A) *Positive clones identified in three expression cDNA libraries from early* Xenopus *embryos. The number on top of each bar indicates the number of proteins isolated as bands by SDS-PAGE in the supernatant of transfected cells. The black portion of the bars indicates unique sequences.* **(B)** *Number of known proteins that have functionally been characterized in* Xenopus*, related proteins characterized in other vertebrates but not in* Xenopus *and novel proteins not characterized yet.* **(C)** *Unique sequences isolated in the individual expression cDNA libraries.* **(D)** *Predicted subcellular localization of cDNAs related to those of other species or to known* Xenopus *cDNAs. Proteins are subdivided into secreted, lysosomal/ endoplasmatic reticulum (ER)/Golgi apparatus and cytoplasmatic/nuclear; numbers of cDNAs identified are indicated.* **(E)** *Subcellular localization of known and related sequences in each cDNA library. M, maternal library (32-cell stage); vG, ventral gastrula stage library, dG, dorsal gastrula stage library.*

determine their identity. Since the three different cDNA libraries were almost equally represented among the total number of clones screened (with the exception of the ventralized gastrula library where clones were picked slightly above average), the overall yield of proteins showing reproducible bands and the proportion of unique sequences could be compared among the individual libraries. We retrieved 33 positive cDNA clones from the maternal library, 59 clones from the ventralized gastrula library and 78 clones from the dorsalized gastrula library. Within both gastrula stage libraries, two out of three isolated cDNAs on average were unique, while in the maternal library only every second sequence was unique. These observations indicated that screening of the gastrula libraries was more fruitful than of the maternally derived library.

We next determined the novelty of the proteins by comparing the sequences against public nucleotide and protein databases (Fig. 2B). Of the 87 unique sequences obtained, 31 clones (36%) were already known *Xenopus* genes, 42 sequences (48%) had a high degree of homology to previously characterized genes and 14 clones (16%) were determined to be novel, i.e. showing little or no homology with sequences of known function. Within each library tested, the frequency of known proteins was lowest in the maternal library and significantly higher in the gastrula stage libraries (Fig. 2C). The percentage of novel proteins was similarly high in the maternal and ventralized gastrula library and relatively low in the dorsalized gastrula library. This result was not surprising, given that rather little attention has been given to *Xenopus* libraries at the cleavage stage, whereas due to the interest in the Spemann-Mangold organizer phenomenon, proteins present in the dorsal gastrula have been the target of numerous screens in the past.

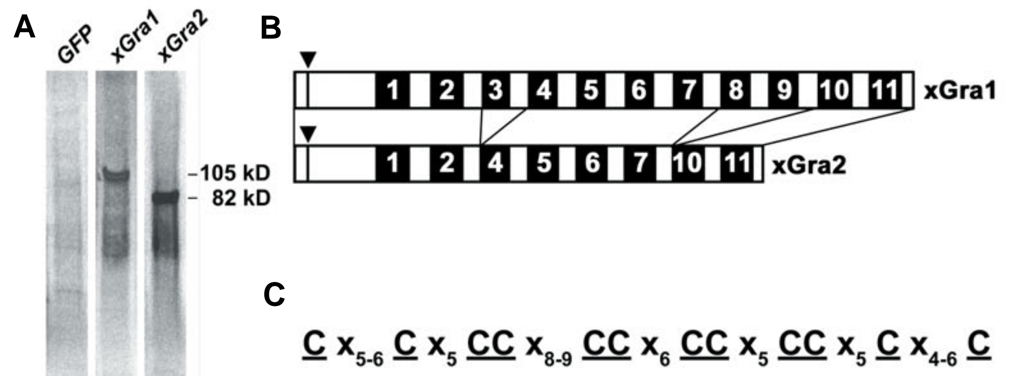
A major criterion for the success of our screen was the amount of secreted proteins among the isolated clones. The predicted subcellular localization of proteins previously characterized in *Xenopus* or other vertebrates is shown in Figure 2D. Table 1 records the known *Xenopus* proteins isolated, Table 2 the clones related to proteins characterized in other organisms and Table 3 the novel proteins identified. Of 152 isolated known *Xenopus* or related sequences from other organisms, 51 clones (34%) were reported as secreted proteins and 56 proteins (37%) were normally localized in lysosomes, endoplasmic reticulum (ER) or the Golgi apparatus. Partial 5' sequences of these clones revealed that each of them contained a signal sequence characteristic of proteins destined for the secretory pathway. It is of interest to note that no plasma membrane-specific protein was isolated. The remaining 45 clones (30%) were cytoplasmic or nuclear proteins and represented false positives, i.e. proteins not expected to be secreted. The high abundance of secreted proteins in our screen indicates that secretion cloning is an efficient method to enrich for extracellular proteins. We then compared the subcellular localization of previously characterized proteins for each library (Fig. 2E). Notably, the percentage of secreted proteins was significantly lower in the maternal library than in the gastrula libraries. Concomitantly, the number of lysosomal, ER and Golgi proteins is relatively high in the library from 32-cell stage embryos. When all proteins are considered (including the novel ones with an authentic signal peptide),

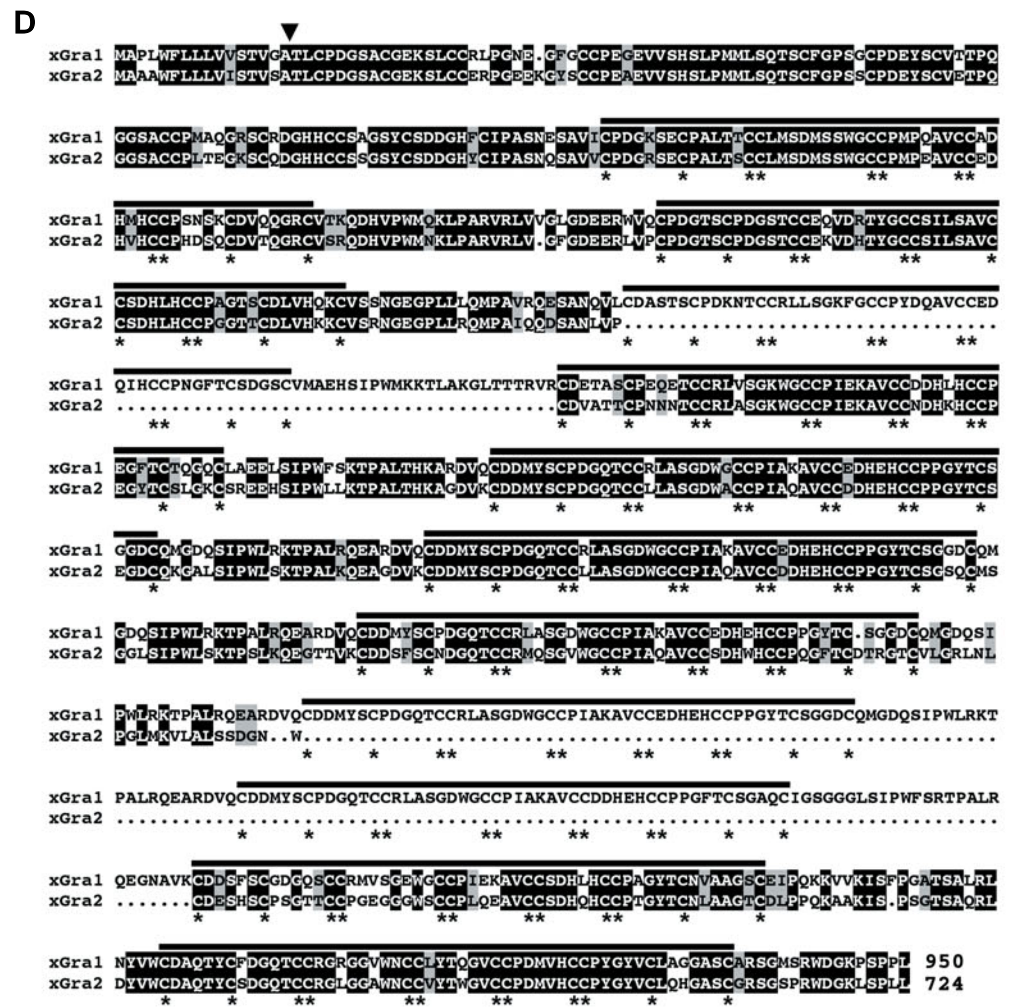
TABLE 1

SUMMARY OF KNOWN *XENOPUS* GENES ISOLATED BY SECRETION CLONING

Name	Family	Activity	No. of isolates	Library Acc. No.	GenBank
Secreted					
Noggin		BMP inhibitor	1	vG	DN828606
Frzb-1	sFRP	Wnt inhibitor	3	vG, dG	DN828607
Sizzled	sFRP	Wnt/BMP inhibitor	5	M, vG	DN828609
Cerberus	Cerberus	Nodal/BMP/Wnt inhibitor	2	dG	DN828648
DAN	Cerberus	BMP inhibitor	1	dG	DN828649
Nodal related-3	TGFβ superfamily	BMP/Activin inh.; FGFR act.	4	dG	DN828650
Antivin/Lefty	TGFβ superfamily	Nodal/Activin inhibitor	2	dG	DN828651
ADMP	TGFβ superfamily	Growth factor	2	dG	DN828652
Glypican-4	Heparan Sulphate Proteoglycan	Wnt/FGF co-receptor	1	dG	DN828653
Lunatic Fringe	Glycosyltransferase	Notch modulator	2	vG	DN828610
Egg Envelope gp37/ZP1			1	vG	DN828611
Cortical Granule Lectin	Eglectin		1	vG	DN828612
Ferritin H			1	dG	DN828654
Lysosomal/ER/Golgi					
Cathepsin D	Asp protease		2	vG, dG	DN828613
TER-ATPase	AAA ATPase	Vesicle formation	7	vG	DN828614
prenylated v-SNARE 1		Vesicle formation	1	dG	DN828655
DG42	Hyaluronan synthase	Glycosaminoglycan synthesis	1	dG	DN828656
Cytoplasmic/Nuclear					
14-3-3 protein zeta	14-3-3 protein	Cell cycle, signal transduction	5	vG, dG	DN828657
14-3-3 protein epsilon	14-3-3 protein	Cell cycle, signal transduction	1	dG	DN828658
14-3-3 like protein	14-3-3 protein	Cell cycle, signal transduction	3	dG	DN828659
Strathmin		Signal transduction	1	M	DN828586
Rho A	Small Rho-like GTPase	Cell adhesion, patterning	1	vG	DN828615
β-Actin		Cytoskeleton	1	vG	DN828616
γ-Actin		Cytoskeleton	1	M	DN828587
Ribonucleoprotein Alb		RNA binding	1	vG	DN828617
Poly(A) binding protein		RNA binding	1	dG	DN828660
Lactate Dehydrogenase B	Oxidoreductase	Glycolysis	1	dG	DN828661
Aldolase C	Frc.-bisphosphate Aldolase	Glycolysis	1	dG	DN828662
Glutamine Synthetase		Amino acid biosynthesis	1	dG	DN828663
RALDH2	Oxidoreductase	Retinoic acid biosynthesis	1	dG	DN828664
XDRP1		Cell cycle	2	vG	DN828618

M, maternal library (32-cell stage); vG, ventral gastrula stage library; dG, dorsal gastrula stage library.





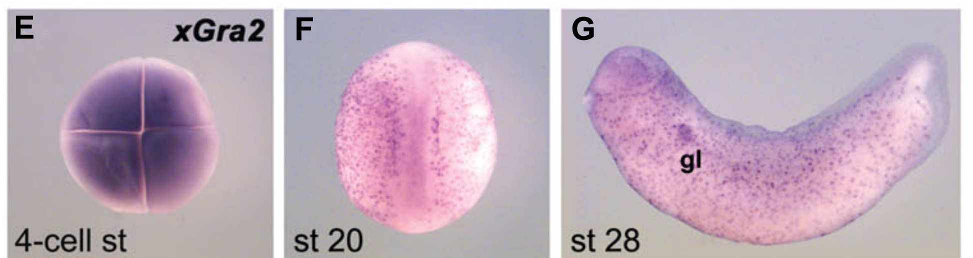


Fig. 3. xGra1 and xGra2 belong to the Granulin family of secreted growth factors. (A) *Supernatant culture medium of human embryonic kidney (293T) cells transfected with cDNA encoding non-secreted green fluorescent protein as control (GFP),* Xenopus *Granulin-1 (xGra1) or* Xenopus *Granulin-2 (xGra2). Cells were labeled with ^{35}S-methionine and -cysteine and their supernatants were analyzed by SDS-PAGE and autoradiography. Note that xGra1 is secreted as a 105 kD protein and xGra2 as an 82 kD protein.* **(B)** *Diagrams of xGra1 and xGra2. The signal peptide cleavage sites are indicated by triangles. The black boxes indicate conserved granulin repeats (Pfam accession number PF00396). The numbers and guidelines indicate repeats conserved in both proteins and are based on sequence similarities. Note that the granulin repeats 3, 8 and 9 of xGra1 are missing in xGra2.* **(C)** *Signature of the granulin repeat. Note the conserved spacing of four cysteine doublets flanked by two cysteine singletons on each side.* **(D)** *Sequence alignment of xGra1 and xGra2. xGra1 corresponds to the Granulin provisional protein sequence previously published for* Xenopus laevis *(GenBank accession number AAH48224) and xGra2 is novel (GenBank accession number DQ004683). Identical amino acid residues are shaded in black and similar or conserved residues in gray. Dots represent gaps introduced into the amino acid sequence in order to obtain optimal alignment. The signal peptide cleavage site as predicted by SignalP is indicated with an arrowhead. Black bars indicate the granulin repeats and the stars the conserved cysteine residues. The overall number of amino acids is indicated at the end of each sequence.* **(E-G)** *Expression of* xGra2 *analyzed by whole-mount* in situ *hybridization.* **(E)** *Four-cell stage embryo in animal view. Note the high level of maternal transcripts.* **(F)** *Embryo at late neurula stage in dorsal view showing spotted expression in the epidermis.* **(G)** *Early tail bud stage embryo in lateral view. Note expression in the pronephric glomus (gl).*

approximately 90 pools (each containing 16 cDNA clones) needed to be screened in order to retrieve one secreted protein. Chances of isolating a secreted protein were highest in the dorsalized gastrula library (one in 55 pools), half as big in the ventralized gastrula library (one in 96 pools) and more than three-fold lower (one in 183 pools) in the 32-cell stage library. These findings may reflect a lower need for extracellular communication at the cleavage stage compared with the intense crosstalk thought to take place between cells in the gastrulating embryo.

Previously characterized Xenopus proteins

Known secreted proteins

Interestingly, the majority of the known secreted proteins turned out to be soluble growth factor antagonists with important roles in development (Table 1). We identified Noggin, a protein that directly induces neural tissue and dorsalizes mesoderm by binding BMPs, preventing them from activating their receptors (Zimmermann *et al.*, 1996). Frzb-1 and Sizzled are putative Wnt antagonists of the same family, that show homology to the extracellular domain of the Wnt receptor Frizzled (Leyns *et al.*, 1997; Salic *et al.*, 1997); subsequent studies suggested that Sizzled also acts by an unknown mechanism on the BMP pathway (Collavin and Kirschner, 2003). We identified the head inducer Cerberus that acts as a multivalent inhibitor of BMP, Wnt and Nodal signals (Piccolo *et al.*, 1999). A Cerberus-related protein, called DAN, binds to BMP and restricts BMP signaling (Hsu *et al.*, 1998). Three distinct members of the transforming growth factor-β (TGFβ) superfamily were identified, two of which may act as antagonists. *Xenopus* Nodal-related-3 (*X*nr3) lacks mesoderm-inducing activity, which is characteristic of other members of the Nodal family and directly induces neural tissue by inhibiting BMP signaling (Hansen *et al.*, 1997); *X*nr3 also regulates convergent-extension movements through the FGF receptor (Yokota *et al.*, 2003). *Xenopus* Antivin (*X*atv, also called Lefty) suppresses mesoderm formation and specifies the left-right axis by antagonizing Nodal signaling (Cheng *et al.*, 2000; Wessely *et al.*, 2001). The Anti-Dorsalizing Morphogenetic Protein (ADMP) is a member of the BMP family, which likely acts as a positive signal through its BMP receptors and inhibits dorsal and anterior development (Moos *et al.*, 1995; Dosch and Niehrs, 2000). The isolation of these proteins strongly supports the view that during early development, an important mechanism for regulating growth factor activity depends on the selective inactivation of signaling pathways by secreted antagonists that block specific ligand/receptor interactions.

We also identified *Xenopus* Glypican-4 (*X*gly-4), which belongs to the family of heparan sulphate proteoglycans linked to the cell surface via a glycosylphosphatidylinositol (GPI) anchor; *X*gly-4 acts as a co-receptor for Wnt and FGF signals and in this way affects convergence-extension movements during gastrulation and dorsoventral patterning of the forebrain, respectively (Ohkawara *et al.*, 2003; Galli *et al.*, 2003). Lunatic Fringe (lFng) was isolated twice. It was originally described as a secreted protein that induces mesoderm formation in *Xenopus* embryos (Wu *et al.*, 1996); recent studies have shown that *lFng* encodes a glycosyltransferase, which modulates Notch signaling (Moloney *et al.*, 2000). Egg Envelope Glycoprotein gp37 constitutes a major component of the egg envelope, an extracellular matrix that surrounds growing oocytes, ovulated eggs and early embryos and is related to the mammalian zona pellucida component ZP1 (Kubo *et al.*, 2000). Cortical Granule Lectin is a calcium-dependent, galactosyl-specific member of the new eglectin family that prevents polyspermy by forming the fertilization layer of the egg envelope (Chang *et al.*, 2004). Finally, heavy-chain Ferritin (Ferritin H) was found; although Ferritin H is primarily a cytosolic protein involved in iron storage, extracellular Ferritin H has been found in blood and other body fluids (Ghosh *et al.*, 2004). The isolation of these known *bona fide* secreted proteins, many of them encoding important signaling factors, is a good indication for the success of the screen. All clones are full-length and can be expressed as transfected DNA, synthetic mRNA or used as *in situ* hybridization probes. They constitute a valuable resource for *Xenopus* researchers.

Known lysosomal/ER/Golgi proteins

In addition, we isolated proteins known to be localized in lysosomes or other compartments of the secretory pathway such as ER or Golgi apparatus. Cathepsin D is an aspartate protease usually restricted to lysosomes (Katz and Taichman, 1999). Seven isolates encoded the transitional endoplasmic reticulum ATPase (TER ATPase), a member of the AAA (ATPases associated with a variety of cellular activities) protein family that contributes to membrane fusion and vesicle formation (Peters *et al.*, 1990). The prenylated vesicle-associated SNARE (v-SNARE) is localized to the ER-Golgi network and plays a role in membrane formation (Park *et al.*, 1998). *Xenopus* DG42 is a ventrally expressed hyaluronan synthetase involved in the synthesis of glycosaminoglycans, which provide a hydrophilic medium in the extracellular space (Rosa *et al.*, 1988; Wessely *et al.*, 2004).

Cytoplasmic and nuclear proteins

In our secretion screen, we also isolated proteins that are normally localized in the cytoplasm or in the nucleus. They usually appeared as weak but distinct bands in the supernatant of transfected and radioactively labeled cells. Cytoplasmic and nuclear proteins may have artificially entered the secretory pathway and in this or by alternative pathways exited the cells, or they may have been released by dying cells. In a few cases, secretion of these proteins has been reported before, such as for members of the 14-3-3 protein family, which normally act as cytoplasmic chaperones for a variety of signaling proteins. We isolated five clones of 14-3-3 protein zeta, a single clone of 14-3-3 protein epsilon and three clones of 14-3-3-like protein in the supernatant of transfected human embryonic kidney 293T cells. 14-3-3 proteins have previously been identified in the medium of cultured keratinocytes (Katz and Taichman, 1999) and in the secretions of parasitic flatworms (Siles-Lucas *et al.*, 2000), suggesting that these proteins may get secreted *in vitro* and *in vivo*.

Sequences homologous to proteins characterized in other species

Related secreted proteins

We have previously presented the gene expression and functional studies on two new members of the secreted Frizzled-related protein (sFRP) family in *Xenopus*, sFRP-2 and Crescent, which add to the growing list of putative growth factor antagonists

TABLE 2

XENOPUS SEQUENCES RELATED TO GENES PREVIOUSLY IDENTIFIED IN OTHER VERTEBRATES

Name	Family	Activity	No. of isolates	Library	Species	GenBank Acc. No.
Secreted						
sFRP-2	sFRP	Wnt inhibitor	3	vG, dG	X. laevis	AF255339
Crescent	sFRP	Wnt inhibitor	1	vG	X. laevis	AF255340
IGFBP-5	IGF binding protein	IGF modulator	1	dG	X. laevis	AY052629
HtrA1	HtrA, Serine protease	BMP/TGFβ inhibitor	2	vG	Mouse	DN828588
Proteinase inhibitor Nexin-1	Ser protease inh. (Serpin)		5	vG, dG	Rat	DN828589
Nucleobindin	EF-hand Ca2+-bindg. prot.		3	M,vG,dG	Rat	DN828619
Cyclophilin B	Peptidyl-prolyl isomerase		2	dG	Human	DN828620
Autotaxin	Exo-Phosphodiesterase		1	dG	Human	DN828621
TCTP	Small chaperon		5	M, dG	Human	DN828622
TRAP-5b	Acid phosphatase		2	dG	Human	DN828623
Lsyosomal/ER/Golgi						
Cathepsin B	Cys protease, papain	Protein metabolism	7	vG, dG	Mouse	DN828590
Cathepsin L	Cys protease, papain	Protein metabolism	19	M,vG,dG	Fish	DN828624
Prosaposin		Lipid met.; neurotroph. fact.	3	vG, dG	Chick	DN828591
α-Mannosidase	Glycosylhydrolase	Carbohydrate metabolism	1	vG	Human	DN828592
β-Hexosaminidase B	Glycosidase	Carbohydrate metabolism	1	M	Mouse	DN828578
CHMP-1		Vesicle trafficking	1	vG	Human	DN828593
Rab GDP dissociation inhibitor	GTPase activating protein	Vesicle trafficking	1	dG	Chick	DN828625
Retinol Dehydrogenase-10	Epimerase	Retinoic acid biosynthesis	1	dG	Mouse	DN828626
Transaldolase		Carb. met., pentose pathway	2	M, vG	Human	DN828594
Ribulose-5-P-3-epimerase	Epimerase	Carb. met., pentose pathway	1	dG	Human	DN828627
EDJ	HSP40, DnaJ	Chaperone	1	dG	Mouse	DN828628
47kD Heatshock Protein	Serpin	Chaperone	1	dG	Mouse	DN828629
Torsin B	AAA ATPase	Chaperone	2	vG	Human	DN828595
Protein Disulphide Isomerase	Oxidoreductase	Chaperone	1	dG	Human	DN828630
ER KDEL Receptor		Protein sorting	1	dG	Human	DN828631
ERGIC-53	Legume-like Lectin	Glycoprotein transport	1	M	Rat	DN828579
VIP36	Legume-like Lectin	Glycoprotein transport	1	dG	Mouse	DN828632
Cytoplasmic/Nuclear						
14-3-3 Protein theta	14-3-3 Protein	Cell cycle, signal transduction	1	dG	Mouse	DN828633
Tropomyosin		Cytoskeleton	1	vG	Rat	DN828596
Protein Phosphatase X	Ser/Thr Prot. Phosphatase	Microtubule nucleation	1	vG	Rabbit	DN828597
Phosphoglycerate Mutase	Mutase	Glycolysis	4	M, dG	Rat	DN828580
Pyruvate Kinase	Phosphotransferase	Glycolysis	3	dG	Chick	DN828634
Fructose-1,6-bisphosphatase	Hydrolase	Gluconeogenesis	1	dG	Human	DN828635
Malate Dehydrogenase	Dehydrogenase	Tricarboxylic acid cycle	1	dG	Cat	DN828636
Serine Hydroxymethyltransferase	Methyltransferase	Amino acid metabolism	2	M, dG	Sheep	DN828637
Guanidinoacetate Methyltransferase	Methyltransferase	Creatine biosynthesis	1	dG	Rat	DN828638
Uroporphrinogen Decarboxylase	Decraboxylase	Heme biosynthesis	1	dG	Fish	DN828639
Adenylosuccinate Lyase	Lyase	Purine biosynthesis	4	M,vG,dG	Chick	DN828598
Aldehyde Reductase-1	Aldo/Keto Reductase		1	vG	Rat	DN828599
Prolidase	Peptidase		1	dG	Mouse	DN828640
SMARC	SWI/SNF	Chromatin remodeling	1	vG	Human	DN828600
Heat shock factor binding protein-1		Transcription factor	1	M	Human	DN828581

For abbreviations, see Table 1.

secreted by the Spemann-Mangold organizer (Pera and De Robertis, 2000). Furthermore, we obtained the *Xenopus* homolog of IGF binding protein-5 (IGFBP5) that promotes head development by stimulating IGF signaling (Pera *et al.*, 2001). We now present a novel *Xenopus* homolog of HtrA1 (Table 2), which encodes a secreted serine protease with an IGF-binding motif (Clausen *et al.*, 2002). In addition, we isolated *Xenopus* Protease Nexin-1 (PN-1), a secreted serine protease inhibitor of the serpin superfamily (Küry *et al.*, 1997). We also isolated a new *Xenopus* homolog of Nucleobindin, a signal peptide containing EF-hand

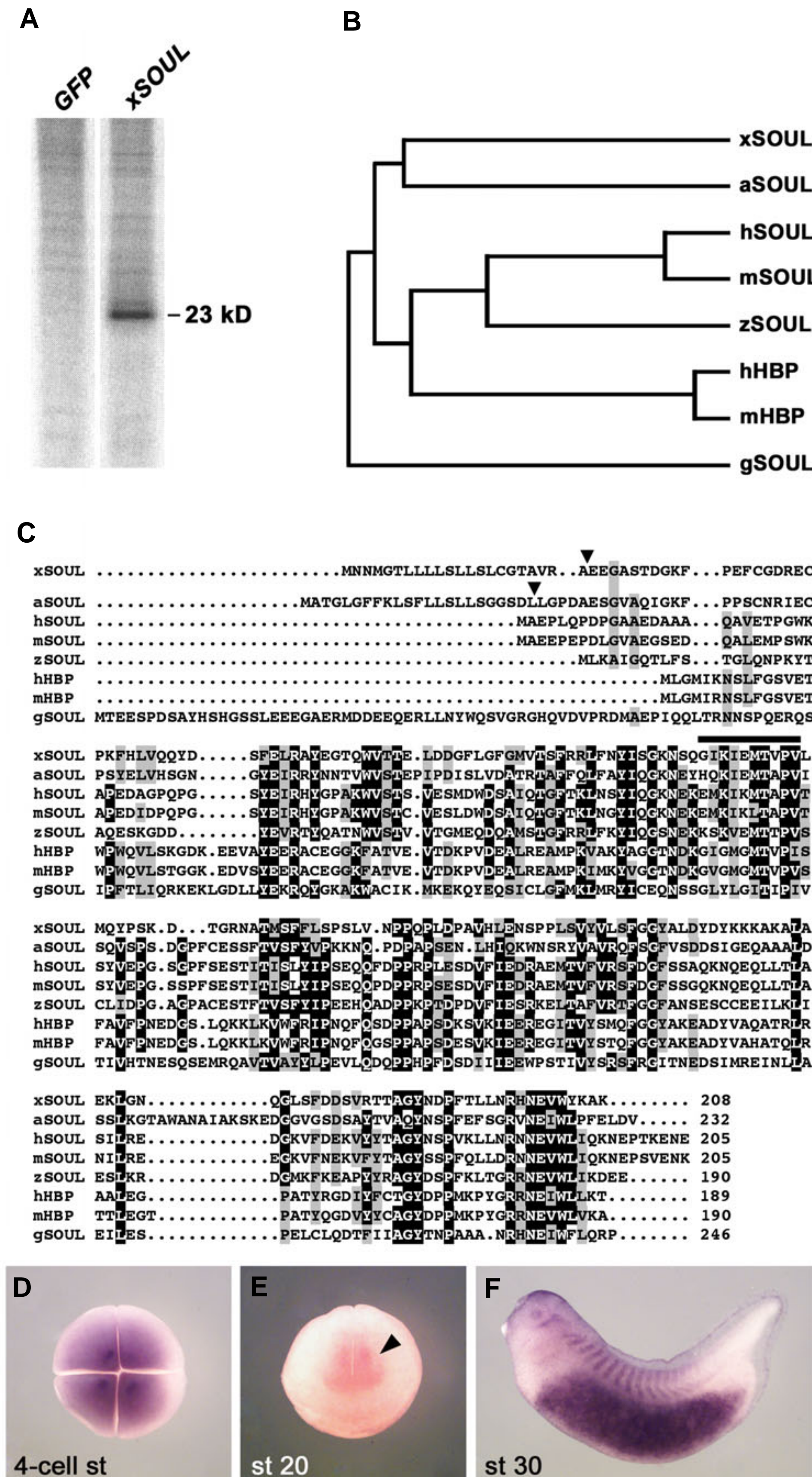


Fig. 4. xSOUL is a novel secreted protein of the SOUL/Heme-binding protein family. (A) *Culture medium of 293T cells transfected with* green fluorescent protein *cDNA as control (* GFP*) or* Xenopus SOUL *cDNA (*xSOUL *). xSOUL is secreted as a 23 kD protein.* **(B)** *Evolutionary relationship of xSOUL and other members of the SOUL/heme binding protein (HBP) family. Proteins with the greatest sequence similarity cluster together and branch lengths are proportional to distance (TreeTop-Phylogenetic Tree Prediction; http://www.genebee.msu.su/services/phtree_reduced.html). Only the mature proteins have been considered. Note that xSOUL is most closely related to a derived family member in the plant* Arabidopsis thaliana *(aSOUL). The listed proteins have the following GenBank accession numbers: xSOUL (GenBank accession number DQ004682); aSOUL (NM101570); human SOUL (AF117616); mouse SOUL (AF117614); zebrafish SOUL (BC045936); human HBP (AF117615); mouse HBP (AF117613); chick SOUL (AF117612).* **(C)** *Comparison of xSOUL with other SOUL/HBP sequences. Note that only xSOUL and aSOUL have cleavable signal peptides (triangle). The putative heme-binding region as reported by Zylka and Reppert (1999) is overlined.* **(D)** *Four-cell stage embryo in animal view showing maternal expression of* xSOUL. **(E)** *Anterior view of advanced neurula. Note weak expression in the anterior brain (arrowhead).* **(F)** *Tailbud stage embryo with distinct expression in the forebrain, lens, somites and gut.*

calcium-binding protein, which is known to be secreted, since the protein has been found in the bone extracellular matrix, blood serum and the supernatant of cultured cells (Lavoie *et al.*, 2002). Another extracellular protein found in our screen was *Xenopus* Cyclophilin B; cyclophilins were initially identified as binding proteins for the immunosuppressive drug cyclosporin A and possess peptidyl-prolyl isomerase activity thought to participate in protein folding and leukocyte chemotaxis (Bukrinsky, 2002). We found a novel *Xenopus* protein related to Autotaxin, also termed tumor cell motility-stimulating protein; this glycoprotein is synthesized as a transmembrane protein, but upon cleavage is released as a soluble phosphodiesterase to activate cellular motility (Murata *et al.*, 1994). Another clone encoded the *Xenopus* homolog of the translationally controlled tumor protein (TCTP); this molecule is highly conserved in eukaryotes, shares similarity with small chaperones and functions in cell cycle progression, malignant transformation and protection against apopto-

sis. TCTP also displays extracellular cytokine-like activities and induces the production of histamine and interleukins (Bommer and Thiele, 2004). A *Xenopus* homolog of the tartrate-resistant acid phosphatase (TRAP) has been isolated; in mammals, TRAP is secreted from osteoclasts into the circulation (Alatalo *et al.*, 2000).

Clones related to lysosomal/ER/Golgi proteins

A large proportion of the *Xenopus* clones that exhibit homology to non-secreted proteins of the secretory pathway encoded cysteine proteases of the Cathepsin family. We isolated seven clones of a protein most similar to Cathepsin B and even nineteen isolates of a protein related to Cathepsin L. Secretion of lysosomal enzymes in tissue culture has been known for a long time (Hasilik, 1992) and members of the Cathepsin family have previously been reported to be secreted by epidermal keratinocytes in culture (Katz and Taichman, 1999). Three other isolates were related to Prosaposin, the precursor of the lysosomal Saposin proteins, which are essential for hydrolysis of glycosphingolipids; Prosaposin has also been suggested as a neurotrophic factor in the brain (O'Brien *et al.*, 1994). We identified two new *Xenopus* homologs of lysosomal glycosidases (α-Mannosidase and β-Hexosaminidase B). α-Mannosidase is required for degradation of asparagine-linked carbohydrates of glycoproteins and its deficiency in human and other mammals results in the lysosomal storage disorder α-mannosidosis (Sun and Wolfe, 2001). Lysosomal β-Hexosaminidases are indispensable for degradation of gangliosides (an essential class of outer-layer membrane lipids) and mutations in the human β-Hexosaminidase B subunit cause the lysosomal storage disorder Sandhoff disease (Maier *et al.*, 2003). The isolation of both enzymes in our secretion screen is not surprising, given that secretion of α-Mannosidase has been reported from cultured cells (Sun and Wolfe, 2001) and β-Hexosaminidase B has been found in human serum (Isaksson and Hultberg, 1995).

We isolated the *Xenopus* homolog of CHMP1 (charged multivesicular body protein-1) that localizes to the endosome and plays a role in vesicle trafficking (Howard *et al.*, 2001). The Rab-GDP-dissociation inhibitor (RabGDI) modulates membrane association of various Rab GTPases, which are key regulators of vesicular protein transport (Bartz *et al.*, 2003). We also cloned the *Xenopus* homolog of Retinol Dehydrogenase-10 (RDH10), which is abundant in microsomes and involved in the synthesis of retinoic acid (Wu *et al.*, 2002). We detected two components of the pentose phosphate pathway in *Xenopus* (Transaldolase, Ribulose-5-phosphate-3-epimerase). Although enzymes of this pathway are cytoplasmic, they have also been localized to the ER of mammals (Bublitz and Steavenseon, 1988). Four *Xenopus* proteins isolated were related to the molecular chaperones EDJ, HSP47, Torsin A and Protein Disulphide Isomerase that are normally found in the lumen of the ER. EDJ (for ER-associated DNAJ) is a heat shock protein of the HSP40 family involved in protein synthesis, folding and secretion (Yu *et al.*, 2000). The 47-kD heat shock protein (HSP47) is a chaperone derived from the serpin family and important for the processing and/or secretion of procollagen (Dafforn *et al.*, 2001). Torsin B is a member of the AAA ATPase family expressed in the ER and is involved in protein transport and degradation; mutations in human Torsin B may lead to early-onset distonia and torticallis, a neurological disorder characterized by abnormal movements (McNaught *et al.*, 2004). The Protein Disulphide Isomerase isolated belongs to the family of oxidoreductases and catalyzes the formation, isomerization and reduction of disulphide bonds (Jessop *et al.*, 2004). In addition, we found the *Xenopus* homolog of the mammalian KDEL endoplasmic reticulum retention receptor that retains soluble ER chaperones with the carboxyterminal Lys-Asp-Glu-Leu (KDEL) sequence in the ER by retrieving them from later stages in the secretory pathway (Yamamoto *et al.*, 2003). We also isolated two *Xenopus* homologs of the Lectin family (ERGIC-53, VIP36). Lectins are non-enzymatic carbohydrate-binding proteins with a

TABLE 3

NOVEL SEQUENCES OBTAINED THROUGH SECRETION CLONING

Name	Family	Putative activity	No. of isolates	Library	Best hit	Species	GenBank Acc. No.	References
Secreted								
Isthmin	Thrombospondin-rel.		1	dG	Isthmin	*X. laevis*	AV091635	Pera *et al.*, 2002
Darmin	Glu-Carboxypeptidase	Zn-Metalloprotease	1	vG	Glu carboxypeptidase Darmin	*X. laevis*	AY166869	Pera *et al.*, 2003
Darmin-r	Glu-Carboxypeptidase	Zn-Metalloprotease	1	M	EST Darmin-related	*X. laevis*	BU993920	Pera *et al.*, 2003
Granulin-1	Granulin/Epithelin	Growth Factor	1	vG	Granulin	*X. laevis*	DN828601	this paper
Granulin-2	Granulin/Epithelin	Growth Factor	1	vG	Hyp. prot. LOC443608	*X. laevis*	DQ004683	this paper
SOUL			5	M, dG	TEgg032m12	*X. tropicalis*	DQ004682	this paper
Xystatin	Cystatin family	Cys protease inhibitor	1	vG	Egg-white cystatin	Quail	DQ004681	this paper
Trinein	Kunitz/Serpin	Ser protease inhibitor	1	dG	Clone S10-40-F7 mRNA	*X. laevis*	DN828641	this paper
ARMET			1	dG	ARMET protein	*X. laevis*	DN828642	this paper
LAMA-like	Laminin A-rel.		1	M	Hyp. prot. LOC196463	Human	DN828582	this paper
Non-secreted								
LZ1601/10			1	vG	Hyp. Prot. MGC78953	*X. laevis*	DN828602	this paper
LZ1665/1			1	vG	TEgg024e02	*X. tropicalis*	DN828603	this paper
LZ2383/11			1	vG	Hyp. Prot. MGC84563	*X. laevis*	DN828604	this paper
OW500/12			1	dG	Hyp. Prot. MGC80796	*X. laevis*	DN828643	this paper

Listed are proteins with no known function. For abbreviations, see Table 1.

role in quality control and protein traffic along the secretory pathway (Hauri *et al.*, 2000). ER-Golgi intermediate compartment (ERGIC)-53 is a mannose-binding type 1 transmembrane protein that operates as a cargo receptor in the transport of glycoproteins from the ER to the Golgi. The related vesicular integral membrane protein (VIP36) may operate in quality control of glycosylation in the Golgi apparatus. Thus our screen identified many components of the secretory pathway in *Xenopus*, several of which regulate the processing, secretion and intracellular transport of proteins.

Novel secreted proteins

Our screen identified ten unique cDNA sequences encoding novel secreted proteins (Table 3). Some of these proteins may have already been deposited as *Xenopus* sequences in public databases, or they may have homolgy to known sequences in other organisms. Nevertheless, we regard them as novel in the sense that they have not been functionally characterized yet. We applied three criteria for classifying a novel protein as secreted. (1) Abundance of a cleavable signal peptide as predicted by SignalP (Bendtsen *et al.*, 2004) or the PSORTII database. (2) Relation to other proteins previously shown to be secreted. (3) Detection of secreted protein in the supernatant of transfected 293T cells as strong band or smear in SDS-PAGE. We have previously presented three novel proteins isolated by secretion cloning. Isthmin is a novel secreted protein with a thrombospondin type1 repeat (Pera *et al.*, 2002). Darmin (Drm) and Darmin-related (Drm-r) are both Glutamate Carboxypeptidases; while Drm contains a predicted signal peptide and is heavily released from transfected cells, Drm-r lacks an apparent signal peptide and is only poorly secreted (Pera *et al.*, 2003). Trinein encodes a serine protease inhibitor of the Kunitz-type (O.W., José Garcia-Abreu, E.M.P and E.M.D.R., manuscript in preparation).

Xenopus ARMET-like exhibits similarity to ARMET (arginine-rich, mutated in early stage tumors), a protein susceptible to mutations in various human cancers (Shridhar *et al.*, 1997). ARMET is highly conserved and homologs in mouse (GenBank accession number NP083379), *Drosophila* (GenBank accession number AAD32615) and *C. elegans* (GenBank accession number NP500273) have cleavable signal peptides and are predicted to be extracellular (PSORT II). We detected *Xenopus* ARMET-like as a strongly secreted protein of 20 kD in the supernatant of cultured cells (data not shown), which matches the predicted molecular weight of *C. elegans* ARMET-like protein (19 kD). The normal function of ARMET is unknown. An additional novel protein shows similarity to the secreted *C. elegans* Laminin A precursor (E-value 2e-30; GenBank accession number NP499668) and *Drosophila* LAMA (lamina ancestor; E-value 3e-18; GenBank accession number AAB49926; Perez and Steller, 1996) and is consequently referred to as LAMA-like. We detected LAMA-like as a 70 kD protein in the supernatant of cultured cells (data not shown), which matches the predicted molecular weights of *C. elegans* Laminin A precursor (65.4 kD) and *Drosophila* LAMA (69 kD, Perez and Steller, 1996).

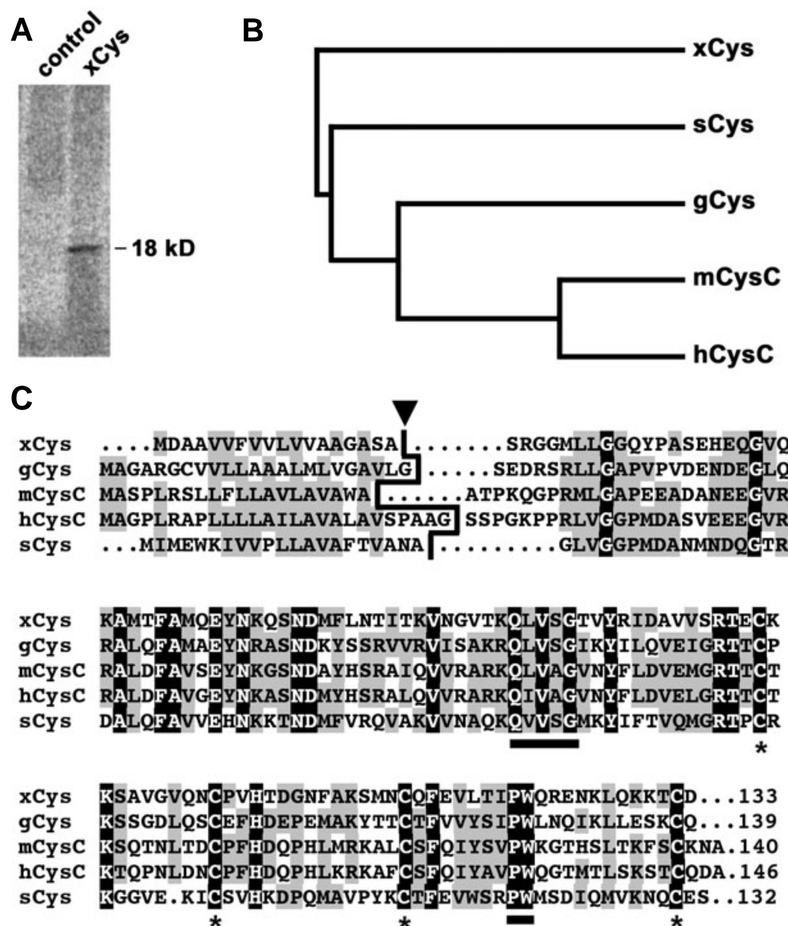


Fig. 5. Xystatin is a novel secreted cysteine proteinase inhibitor. (A) *Supernatant of 293T cells non-transfected (control) or transfected with* Xenopus Cystatin *cDNA (*xCys*). Note that xCys is secreted as an 18 kDa protein.* **(B)** *Phylogenetic tree indicating the relationship of xCys with other members of the cystatin superfamily. Only the mature proteins lacking the signal peptide have been aligned. GenBank accession numbers are as follows: Xystatin (DQ004681); salmon Cystatin (D86628); chick Cystatin (JO5077); murine Cystatin-C (NM009976); human Cystatin-C (BC013083).* **(C)** *Sequence alignment of Xcys and related members of the cystatin superfamily. Four conserved cysteine residues known to form two disulphide bonds are labeled with stars. The conserved active site sequence QxVxG and pro-trp (PW) sequences are underlined (Brown and Dziegielewska, 1997).*

Two novel growth factors of the Granulin family in Xenopus

We found two putative growth factors of the granulin or epithelin family, termed *Xenopus* Granulin (xGra)-1 and xGra-2 (Fig. 3). Granulins are candidate growth factors discovered in human leukocytes. They exhibit both proliferative and antiproliferative effects on epithelial cell lines. Members of this family share structural similarity with epidermal growth factor (EGF) and TGFα. Both Granulin and EGF are cysteine-rich peptides of approximately 6 kD. Although dissimilar in their amino acid sequence, they show a common tertiary structure. The precursors of mammalian granulins and EGF are both organized as tandem

repeats of conserved cysteine modules. The 6 kD peptide forms, the intact precursor and the related TGFα protein regulate cell growth (Bhandari *et al.*, 1992; Bateman and Bennett, 1998).

xGra1 and xGra2 were strongly secreted as intact precursors by transfected 293T cells, with xGra1 exhibiting a molecular weight of 105 kD and xGra2 of 82 kD (Fig. 3A). *xGra1* is identical to a cDNA sequence, which has been previously deposited in the NCBI database (GenBank accession number BC048224) and encodes a protein of 950 amino acids with eleven copies of the granulin domain (Fig. 3B, upper panel). The novel *xGra2* sequence yields a smaller protein (724 amino acids) with only eight granulin repeats (Fig. 3B, lower panel). The granulin domains of xGra1 and xGra2 show the characteristic spacing of 12 cysteines with four cysteine doublets flanked by two singletons, as indicated in Fig. 3C. The alignment of the two proteins reveals a conserved signal peptide cleavage site (between positions 16 and 17) and an overall amino acid identity of 76% (Fig. 3D). Closer examination of the amino acid sequences indicates that among the 11 granulin repeats present in xGra1, the granulin repeats 3, 8 and 9 are missing in xGra2 (Figs. 3 B,D).

We analyzed the expression of the novel *xGra2* gene during early *Xenopus* development by whole-mount *in situ* hybridization. A strong signal was detected at the 4-cell stage, indicating abundant maternal transcripts (Fig. 3E). From neurula stage onwards, we found spotted expression in the epidermis (Fig. 3F). At tail bud stage, an additional expression domain appeared in the pronephros anlage (Fig. 3G). The spotted expression pattern of *xGra2* is similar to that of *α-Tubulin*, which demarcates ciliated cells in the epidermis and is regulated by Notch-signaling (Deblandre *et al.*, 1999).

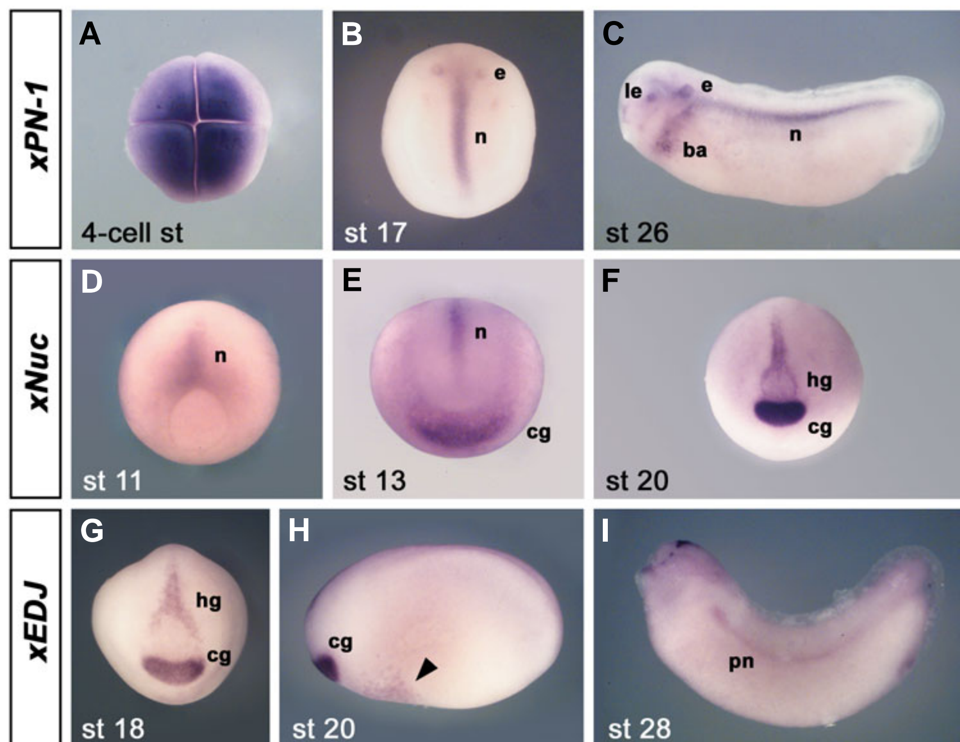


Fig. 6. Whole-mount *in situ* hybridization of xPN-1, xNuc and xEDJ. *Embryos are shown in animal (A), dorsal (B,D), lateral (C,H,I) or anterior view (E-G).* **(A-C)** Xenopus *P*roteinase Nexin-1 (xPN-1). **(A)** *Four-cell stage embryo showing high level of maternal transcripts.* **(B)** *Late neurula. Note expression in the notochord (n) and ear placode (e).* **(C)** *Early tail bud stage with additional expression domains in the lens (le) and ectoderm of the branchial arches (ba).* **(D-F)** Xenopus Nucleobindin (xNuc). **(D)** *Mid-gastrula showing expression in the notochord (n).* **(E)** *Early neurula with additional signal in the cement gland (cg).* **(F)** *Late neurula. Note strong expression in the cement gland and hatching gland (hg).* **(G-I)** Xenopus ER-associated DNAJ chaperone (xEDJ). **(G)** *Late neurula with expression in cement gland (cg) and hatching gland (hg).* **(H)** *Late neurula depicting faint signal on the ventral side (arrowhead).* **(I)** *Early tail bud stage. Note expression in the pronephros (pn) and in the hatching gland in the dorsal head.*

Xenopus SOUL is a novel secreted protein

We identified a new member of the SOUL/heme-binding protein (HBP) family, designated xSOUL (Fig. 4). The name was given due to sequence similarities to chick SOUL, which has expression in the pineal gland, the organ René Descartes hypothesized was the location of the soul (Zylka and Reppert, 1999). We found five isolates of xSOUL with a molecular weight of 23 kD in the maternal and dorsal gastrula stage cDNA libraries (Table 3, Fig. 4A). Partial sequencing revealed that these clones represent at least three distinct pseudoalleles with significant differences in the 5'UTR (data not shown). One cDNA clone selected for full-length sequencing encodes a protein of 208 amino acids. Data base comparisons showed related proteins in human, mouse, zebrafish, chick and plants (Fig. 4B). The *Xenopus* protein is highly divergent, since the closest relative was the SOUL-related protein from *Arabidopsis* (35% amino acid identity). It is of interest to note that only *Xenopus* and *Arabidopsis* SOUL contained cleavable signal peptides (Fig. 4C). Indeed, the mouse SOUL and HBP proteins were found to be cytoplasmic (Zylka and Reppert, 1999). This suggests that the new *Xenopus* homolog may be unique among vertebrate SOUL/HBP proteins in participating in the secretory pathway. Like other members of this family, xSOUL showed a conserved hydrophobic region, which may be involved in binding of heme or other porphyrin compounds (Zylka and Reppert, 1999). By whole-mount *in situ* hybridization, we detected abundant maternal transcripts of *xSOUL* at the 4-cell stage (Fig. 4D). At late neurula, weak expression was restricted to the midbrain and the posterior part of the forebrain (Fig. 4E). At tail bud stage, multiple expression domains were apparent in the brain, facial mesenchyme, lens, somites and particularly in the gut (Fig. 4F).

A novel cysteine proteinase inhibitor: Xystatin

We identified a new cysteine proteinase inhibitor protein of the cystatin superfamily, designated Xystatin. Xystatin (from *Xenopus* cystatin) was found as an 18 kD protein in the supernatant of

transfected 293T cells (Fig. 5A). Sequence comparison showed that Xystatin is distantly related to cystatin proteins in other vertebrates (Fig. 5B). The full-length cDNA clone encodes a 133 amino acid protein containing an amino-terminal signal peptide typical for secreted proteins (Fig. 5C). The mature protein (115 amino acids) is most homologous to Japanese quail egg white cystatin (38% identity; Brown and Dziegielewska, 1997). Xystatin shares the basic structure of cystatin family members, including the active site QxVxG sequence, a conserved Pro-Trp (PW) sequence and four conserved cysteine residues crucial for the formation of two disulphide loops, making it likely that Xystatin is a functional cysteine protease inhibitor. By whole-mount *in situ* hybridization, we detected maternal transcripts at the 4-cell stage and ubiquitous expression at the gastrula and neurula stage; in tailbud embryos, *Xystatin* mRNA was restricted to the neural tube and neural crest cells in the anterior head and branchial arches (data not shown).

Expression of additional genes identified by secretion cloning

Xenopus *Protease Nexin-1*

Protease Nexin-1 (PN-1) is a serine protease inhibitor of the serpin family (Patston *et al.*, 2004), which so far has only been cloned in amniotes. Embryonic expression has been reported for mouse (Küry *et al.*, 1997) and chick (Rodríguez-Niedenführ *et al.*, 2003) development. In *Xenopus*, *PN-1* is abundantly expressed at the 4-cell stage as maternal mRNA (Fig. 6A). At neurula stage, distinct expression can be detected in the notochord and the otic placodes (Fig. 6B). At tail bud stage, additional expression is seen in the branchial arches, the midbrain-hindbrain boundary (MHB) and the lens (Fig. 6C). In the mouse, it has recently been shown that the MHB expression of *PN-1* is activated by FGF signaling (Küry *et al.*, 1997). Since *PN-1* shares several expression domains with *FGF8*, including the notochord, otic placodes, branchial arches and the MHB, it is possible that *PN-1* may belong to the *FGF8* synexpression group (Niehrs and Meinhardt, 2002).

Xenopus *Nucleobindin*

Nucleobindin (Nuc) and the related NEFA protein (DNA binding/ EF-hand/acidic amino acid rich region) contain a signal peptide for secretion, a region rich in basic amino acids that is presumably a DNA-binding motif, two EF-hand motifs involved in calcium-binding and a leucine-zipper motif that is a dimerization domain (Otte *et al.*, 1999; Lavoie *et al.*, 2002). Although they have been reported to be involved in autoimmunity, apoptosis and calcium homeostasis in the Golgi apparatus and bone matrix, their exact role remains unknown. *Xenopus Nuc* shows dynamic expression during early development. During gastrulation, we detected transcripts at the dorsal blastopore lip and the embryonic midline (Fig. 6D). At the neural plate stage, expression was maintained along the dorsal midline and a new domain arose anteriorly in the cement gland primordium (Fig. 6E). We also detected weak staining in the epidermis. With the closure of the neural tube, strong signals were restricted to the cement gland and hatching gland (Fig. 6F). Interestingly, the *Drosophila* homolog *nucb1* shows distinct expression in the salivary glands and their placodes (Otte *et al.*, 1999), suggesting that the NEFA-nucleobindin class of calcium-binding EF hand proteins may have an evolutionarily conserved function in secretory gland tissues.

Xenopus *EDJ*

We identified the *Xenopus* homolog of the ER-associated DNAJ (EDJ) protein. EDJ is a member of the heat shock protein 40 (Hsp40) family that acts as a molecular chaperone in the lumen of the endoplasmic reticulum and as such is involved in translocation and degradation of misfolded proteins (Yu *et al.*, 2000). By whole-mount *in situ* hybridization, we detected abundant maternal RNA of *Xenopus EDJ (XEDJ)* at the 4-cell stage and ubiquitous expression at the gastrula stage (data not shown). At late neurula stage, *XEDJ* transcripts were restricted to the anterior cement gland and hatching gland anlage (Fig. 6G). When the embryo started to elongate, further expression appeared ventrally in the area of the liver (Fig. 6H). At advanced tailbud stage, an additional expression domain was found in the pronephros (Fig. 6I).

Discussion

To gain a more comprehensive understanding of intercellular signaling events, we set out to explore the molecular composition of the extracellular space in the early frog embryo. We developed a new technique to directly isolate secreted proteins as full-length cDNA clones. Our secretion cloning method was without bias, i.e., proteins were detected merely by the fact that they were secreted by transfected cells and labeled with ^{35}S-Methionine or ^{35}S-Cysteine. This retrieval of proteins irrespective of their function allowed us to identify new signaling proteins. By screening three expression cDNA libraries from early *Xenopus* embryos, we could isolate 170 positive clones. Among them, 51 encoded *bona fide* secreted proteins that were previously characterized in *Xenopus* or other vertebrates. Another 14 clones encoded novel secreted proteins. The results demonstrate that secretion cloning is a fruitful approach that provides an important method to investigate cell-cell communication during development.

Secretion cloning provides a direct way to isolate secreted proteins

Previous screens for secreted proteins were primarily directed at the presence of an amino-terminal signal peptide that allows the entry of the protein into the secretory pathway (Klein *et al.*, 1996; Skarnes *et al.*, 1995; Tashiro *et al.*, 1999). In these signal sequence trap approaches, cDNA fragments provide a signal peptide to a carboxy-terminally fused protein. A mayor caveat of this screening technique was that the isolated cDNA clones were only partial. Our secretion cloning approach offers the advantage that secreted proteins are synthesized as full-length under native conditions, thereby preserving their structural integrity. The selection for full-length cDNA clones could be explained by the quality-control system in the endoplasmic reticulum, which ‚proof-reads' newly synthesized proteins and targets misfolded proteins to degradation (Ellgaard and Helenius, 2003). Truncated proteins may not acquire the correctly folded conformation because of improper disulphide bond formation or glycosylation and hence fail to be secreted. Since we harvested proteins from the medium of transfected cells, no transmembrane proteins should be detected, which indeed was the case. The absence of plasma

membrane proteins is in striking contrast to previous signal peptide screens, which targeted both secreted and transmembrane proteins. However, we could not prevent picking clones encoding lysosomal, ER and Golgi proteins (Fig. 2, Tables 1 and 2), probably due to the fact that the overexpression of proteins in transfected cells over-saturated the secretory pathway compartments, thus leading to an artificial release into the medium. Release of lysosomal proteins into the supernatant is a common phenomenon in cell culture and triggered for instance by pH changes in the medium (Hasilik, 1992). Lysosomal, ER and Golgi proteins have also been trapped by previous signal peptide screens (Klein *et al.*, 1996; Skarnes *et al.*, 1995; Tashiro *et al.*, 1999).

Many extracellular signaling factors have cysteine-rich domains. Due to the non-reducing milieu in the secretory pathway compartments, covalent disulphide bridges can form before the proteins are released into the extracellular space. Intramolecular disulphide bridges are characteristic of the cystine knot, a structural motif that is frequently found in extracellular proteins (De Robertis and Kuroda, 2004). In our screen, the identification of cystine knot-containing proteins was facilitated through their incorporation of exogeneously added ^{35}S-Cysteine during metabolic labeling. Examples of proteins with a cystine knot motif isolated by secretion cloning are Noggin, Cerberus and members of the TGFβ superfamily (Table 1). In addition, the two novel members of the Granulin family contain multiple copies of the so-called Granulin repeat characterized by twelve regularly spaced cysteine residues (Fig. 3). We note that cysteine-rich domains of the type present in Chordin (CR repeats) were not isolated. These modules bind members of the TGFβ and BMP family (Larraín *et al.*, 2000) and are subjected to regulation by metalloprotease degradation.

Growth factors are normally not freely diffusible but rather bind to the cell surface or extracellular matrix. Recent studies in *Drosophila* and vertebrates have elucidated a crucial role for heparan sulphate proteoglycans (HSPGs) in regulating the distribution of these signaling molecules (Lin, 2004). HSPGs are cell-surface and extracellular matrix molecules composed of a core protein with covalently linked glycosaminoglycan (GAG) chains. The HSPGs interact with members of the Wnt, Hedgehog, TGFβ and FGF family and their GAG chains are of particular importance for the binding to the ligands. In order to compete with this binding and release growth factors, we added Heparin (a mixture of soluble heparan sulphate GAG chains) to the culture medium. However, only few growth factors were isolated in our screen, including three known members of the TGFβ superfamily (Nodal-related-3, Antivin/Lefty, ADMP; Table 1) and the two novel Granulin proteins (Table 3). Despite the large number of cDNA clones screened, many other growth factors normally expressed at the analyzed stages escaped our detection, suggesting that addition of Heparin was not sufficient to release them into the medium. Cell surface and extracellular matrix proteins other than HSPGs may bind these growth factors and restrict their diffusion.

Low yield of secreted proteins in Xenopus *embryos at the 32-cell stage*

Relatively little is known about cell-cell signaling during the first hours of development, when no transcription occurs and all proteins are synthesized from maternal mRNA deposited in the egg. Despite some progress in recent years, the signals and pathways involved are only partially understood (De Robertis *et al.*, 2000). Given that more than twenty-five thousand cDNA clones of the 32-cell stage cDNA library were screened, surprisingly few extracellular proteins with known signaling function were isolated. Among the growth factor antagonists, the only protein we found was Sizzled (Salic *et al.*, 1997; Table 1). Other proteins previously reported to be secreted and not yet known in *Xenopus* included the calcium-binding EF-hand protein Nucleobindin and the translationally controlled tumor protein (TCTP; Table 2). In the unamplified maternal library, we isolated TCTP independently as three different cDNA clones suggesting a high abundance of this gene product in the *Xenopus* egg. A function for Sizzled, Nucleobindin and TCTP at this early stage of development remains to be shown. In addition, we identified the novel secreted proteins LAMA-like, SOUL and Darmin-related (Table 3). Their function remains to be determined. The relatively low yield of secreted signaling proteins suggest that cell-cell communication may not play a major role before mid blastula transition (MBT). It has previously been shown that pre-MBT cell contacts are dispensable for the expression of endodermal and dorsal mesodermal markers (Wylie *et al.*, 1996; Yasuo and Lemaire, 1999). Thus, our data support the notion that early development up to MBT relies more on cell-autonomous signals rather than communications exchanged between cells.

A large variety of signals present at the gastrula stage

The complexity of signals identified rises by the time the embryo undergoes gastrulation. Concomitant with the formation of the three germ layers, cells constantly change their position with respect to each other, while exchanging messages. These intense cell and tissue interactions drive morphogenetic movements and pattern formation in the early embryo. To learn more about the signaling molecules involved, we screened two mid-gastrula stage libraries, one from embryos ventralized by UV exposure and the other from embryos dorsalized by LiCl treatment. We identified a wide spectrum of proteins acting at various levels in the regulation of growth factor signaling. Among the secreted proteins previously characterized in *Xenopus* or other vertebrates, 23 unique sequences were found (Tables 1 and 2). The list comprises one putative growth factor that signals through its own receptor (ADMP), ten soluble modulators that bind to growth factors or their receptors, one serine protease (HtrA1), one serine protease inhibitor (PN-1), five protein-modifying enzymes (including the glycosyltranferase Lunatic Fringe), four proteins of the extracellular matrix (among them the co-receptor Glypican-4) and one protein with transport function (Ferritin H). The majority of these proteins have well-established roles in the control of growth factor signaling in the extracellular space. In addition, we identified eight novel secreted proteins from gastrula-stage embryos (Table 3), including two members of the Granulin/Epithelin family of growth factors (xGra-1 and xGra-2), the metalloprotease Darmin, two putative proteinase inhibitors (Xystatin, Trinein) and proteins of unknown function, such as Isthmin, SOUL and ARMET. It will be interesting to determine their role during gastrulation. Our prelimary studies indicate that the secreted serine protease HtrA1 has important patterning activities in the *Xenopus* embryo (S. H. and E. M. P., data not shown). The serine protease inhibitor Trinein is also active in early devel-

opment (O.W., José Garcia-Abreu, E. M. P. and E. M. D. R., data not shown). Thus cell-cell signaling is not restricted to growth factors and their receptors, but requires an intricate network of cofactors that orchestrate their activity in the extracellular space.

The Spemann-Mangold organizer is a source of secreted antagonists

Our study strongly supports the notion that the Spemann-Mangold organizer is a source of secreted growth factor antagonists. Upon LiCl-treatment, which expands the Spemann-Mangold organizer tissue and dorsalizes the embryos, several antagonists were identified (Table 1 and 2). The isolated factors mediate different functions of the Spemann-Mangold organizer and thereby affect pattern formation and morphogenesis of the embryo. BMP antagonists such as Noggin and *X*nr3 act as direct neural inducers and exert dorsalizing activity (Zimmerman *et al.*, 1996; Hansen *et al.*, 1997). The Wnt antagonists Frzb-1, sFRP-2 and Cerberus along with the pro-IGF signal IGFBP5 stimulate head development (Leyns *et al.*, 1997; Piccolo *et al.*, 1999; Pera and De Robertis, 2000; Pera *et al.*, 2001). In contrast, the putative growth factor ADMP, isolated from LiCl-treated libraries, functions in the trunk organizer and antagonizes head formation (Moos *et al.*, 1995; Dosch and Niehrs, 2000). Antivin/Lefty is involved in determining the left-right axis (Cheng *et al.*, 2000). Finally, three factors regulate convergent-extension movements, i.e. Crescent and Glypican-4 possibly through inhibition of non-canonical Wnt ligands (Pera and De Robertis, 2000; Ohkawara *et al.*, 2003) and *X*nr3 via the FGF type 1 receptor (Yokota *et al.*, 2003). Taken together, our data indicates that growth factor antagonism plays an important role in the organizing centre responsible for the establishment of the vertebrate body plan.

Do lysosomal proteins act in the extracellular space?

Although lysosomal enzymes usually segregate from other secretory proteins in the Golgi apparatus, where they are bound by mannose-6-phosphate receptors and directly routed to the lysosomes, the same receptors may localize to the plasma membrane in order to internalize extracellular lysosomal enzymes through endocytosis (Dahms *et al.*, 1989; Neufeld, 1991). Lysosomal enzymes are generally synthesized as pre-pro-polypeptides and only when they reach the lysosomes they are processed into mature proteins. In our screen, all lysosomal proteins isolated in the supernatant of transfected 239T cells, including Cathepsin B, L and D, Prosaposin, α-Mannosidase and β-Hexosaminidase B, were detected with a molecular weight corresponding to their precursor forms (data not shown), suggesting that they have never entered the lysosome prior to their release to the extracellular environment. Interestingly, all lysosomal enzymes identified by secretion cloning were heavily enriched in the conditioned medium (data not shown) and Cathepsins were the prevalent group of proteins isolated in our screen (with Cathepsin L reoccuring nineteen times; Table 2). These findings suggest that lysosomal proteins may not only act in lysosomes, but also fulfill an independent role in the extracellular space. Indeed, secreted lysosomal enzymes are crucial for tissue and bone remodeling during development (Dahms *et al.*, 1989). Prosaposin, the precursor of four glycoprotein activators (termed saposins A-D) with glycosphingolipid hydrolase activity, has been identified as a neurotrophic factor capable of inducing neural differentiation and preventing cell death in mammals (O'Brien *et al.*, 1994). It will be interesting to determine whether the lysosomal enzymes isolated by secretion cloning fulfill an extracellular function in *Xenopus*.

Secreted proteins as potential targets for therapeutic drugs

In the past, several large-scale efforts have been undertaken to identify new extracellular proteins. The Secreted Protein Discovery Initiative (SPDI) for example used various biological and computational approaches, including the signal sequence trap method in yeast, algorithms for signal sequence prediction and homology-based strategies, to screen for secreted and transmembrane proteins in humans (Clark *et al.*, 2003). Our secretion cloning method complements these efforts and provides a new entry point to further isolate secreted proteins. Although applied here for *Xenopus*, our technique can be used to mine cDNA libraries of any source. In this way, large-scale screens for secreted proteins not only improve our understanding of intercellular communication, but also may lead to new understanding of human diseases and offer possible ways for the development of therapeutic cures. Secreted proteins in particular have properties that lend themselves to be utilized as therapeutic agents or targets. They are accessible to various drug delivery mechanisms, because they are presented within the extracellular space. A purified secreted protein can be utilized directly as a therapeutic, or may be targeted by specific antibodies or small molecules. The novel *Xenopus* proteins that have been identified by secretion cloning and described in this study await further characterization. We note that the early frog embryo provides a practical experimental system to investigate their activity and biological role.

Materials and Methods

cDNA library construction

Three plasmid libraries were screened, in which oligo dT primed cDNAs were cloned unidirectionally into cytomegalovirus (CMV) promoter-based expression vectors. An unamplified library from *Xenopus* UV-ventralized stage 11 embryos in the pcDNA3 vector and TOP10F' bacteria (Invitrogen) was generously provided by Dr. L. Zon (Boston). Two unamplified libraries from *Xenopus* stage 6 and LiCl-dorsalized stage 11 embryos were prepared by inserting cDNA into the *Eco* RI and *Xho* I sites of the pCS2+ vector (a gift of D. Turner) and transforming the clones into XLBlueMRF' bacteria (Stratagene).

Secretion cloning method

Bacterial colonies were manually picked from agar plates and inoculated in 1 ml Terrific-broth/Ampicillin in 96-well blocks (Corning Costar, Cat. -No. 431139). The 96-well plates were covered with Parafilm and shaken for about 24 hours at 37°C and 200 rpm in a humidified incubator. Aliquots from the media of 16 clones (125 μl each) were pooled and plasmid DNA extracted by Miniprep (Qiagen). Human embryonic kidney 293T cells (ATCC) were cultured in 24-well plates using DMEM and 10% fetal calf serum. At 50-70% confluence, 12.5 μM chloroquine was added to inhibit degradation of transfected DNA by lysosomal hydrolysases. The cells were transfected with 2 μg/ml plasmid DNA and 0.2 μg pAdvantage DNA (Promega) using the calcium phosphate method. After 36 hours, the cells were labeled with 110 μCi/ml ^{35}S-Methionine and ^{35}S-Cysteine (Easy Tag Express ^{35}S protein labeling mix, NEN) in serum-free medium. Heparin (50 μg/ml) was added to facilitate the release of secreted proteins from the cell surface. After 36 hours, the medium was harvested in microfuge tubes and cleared from cells by centrifugation at 800 g for 10 min at 4ºC. An aliquot of the medium was loaded onto a precast 4-20%

gradient SDS polyacrylamide gel (Biorad) and electrophoresed under reducing conditions. The gels were fixed in 10% acetic acid and 30% glycerol for 15 min, dried and exposed to a phosphor screen (Molecular Dynamics) or X-OMAT (AR) film (Kodak).

Sib selection and gene identification

Positive clones were individualized in a second step by sib selection. The 16 clones from each positive pool were regrown in a matrix of 4 rows and 4 columns in 96-well arrays. Media from each row and column of the 96-well block were subpooled, plasmid DNA prepared and retransfected. If two subpools of one row and one column generated proteins of the same size as that of the original pool, a positive clone was identified. The selection was validated in a third round of transfection using the individual plasmid DNA. Partial gene sequence of the positive clones was obtained using automated sequencing. Approximately 500 bp of 5' sequence (and eventually 3' sequence) was used to perform BLAST searches of the NCBI nucleotide and protein databases. Names were assigned to the cDNA clones according to the name of the gene with the best hit.

RNA **in situ** *analysis*

Whole-mount *in situ* hybridization was performed as described (http://www.lifesci.ucla.edu/hhmi/derobertis/index.html). For antisense RNA probes, the pcDNA3 constructs of *xGra-2* and *xPN-1* were linearized with *Bam* HI and *Hind* III, respectively and transcribed with Sp6 RNA polymerase. The pCS2+ constructs of *xSOUL*, *xNuc* and *xEDJ* were linearized with *Bam* HI and transcribed with T7 RNA polymerase.

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