Two different precursors for secretory polypeptides from the stomach of *Xenopus laevis* have been characterized by cDNA cloning. Both mature polypeptides are potential candidates for gastrointestinal growth factors. One, xP1, is the *X. laevis* homologue of the pS2 gene product consisting only of a single P-domain, whereas the second, xP4, is a novel polypeptide formed by four P-domains arranged in tandem. Northern analysis detected both transcripts in the stomach but not in the skin or the brain. In *in situ* hybridizations localized the expression of both precursors in surface mucous cells of the gastric mucosa. With an antibody generated against the deduced C-terminal end of xP4, the mature polypeptide was investigated by Western analysis revealing N-glycosylation of xP4.

The term "P-domain" was proposed for a family of characteristic polypeptide sequences consisting of about 50 amino acid residues (Tomasetto et al., 1990). The main characteristic of this domain is the conservation of 6 cysteine residues and a single tryphtophan. Variable numbers of this motif have been discovered in different polypeptides. The human pS2 gene product consists only of a single P-domain (Jakolew et al., 1984), whereas spasmolytic polypeptides from various species (PSP, mSP, hSP; Thim et al., 1985; Rose et al., 1989; Tomasetto et al., 1990) are formed by two such modules arranged in tandem. Additionally, this motif has also been detected in an astonishing variety of high *M*$_r$ proteins with multidomain structures. Known examples are integumentary mucin A1 (FIM-A.1; four P-domains) from *Xenopus laevis* (Hoffmann, 1988; Hauser et al., 1990), the sucrase-isomaltase complex of the small intestinal brush-border membrane, lysosomal α-glucosidase (Tomasetto et al., 1990), and α/β from *X. laevis* skin secretions (Gmachl et al., 1990). Furthermore, P-domains have some similarity with kringle domains of proteins mainly involved in blood coagulation (Baker, 1988).

So far, the molecular function of P-domains in the high *M*$_r$ proteins is unknown. Generally, interaction with other proteins might be expected as discussed for specific kringle domains (Petersen et al., 1990) or epidermal growth factor (EGF)-like repeats (Appella et al., 1988). Alternatively, interaction with carbohydrate moieties is worth considering since P-domains are either part of sugar-degrading enzymes or they are synthesized from mucus-secreting epithelial cells. However, the polypeptides consisting solely of P-domains (pS2 and the PSP group) should be ideal for functional investigations.

The pS2 transcript was originally detected in the MCF-7 breast cancer cell line after estrogen induction (Masiakowski et al., 1982), but not in normal breast tissue. Subsequently, this peptide is rediscovered in MCF-7 cells by screening for EGF-like immunoreactivity (Mori et al., 1988). Specific expression occurs in normal stomach mucosa cells in an estrogen-independent fashion (Rio et al., 1988a). This expression may be regulated by EGF or a similar growth factor since the 5' upstream region of the pS2 gene contains an enhancer region responsive to estrogens and EGF (Nunez et al., 1989). PSP was discovered first in porcine pancreas during the purification of insulin (Jorgensen et al., 1982). PSP secretion is under parasympathetic control (Rasmussen et al., 1990), and it is correlated with the exocrine function of the pancreas (Thim et al., 1982). Expression of PSP, mSP, and hSP transcripts has been described in the stomach and the pancreas of the three species, but to different extents (Tomasetto et al., 1990). PSP has been reported to inhibit gastrointestinal motility and gastric acid secretion (Jorgensen et al., 1982), and receptor binding of PSP to two different sites has been described in rat intestinal mucosa cells (Frandsen et al., 1986; Frandsen, 1988). Also, a role as an autocrine growth factor has been suggested due to the growth-stimulatory effect of PSP on cultured colon and breast tumor cells (Høosein et al., 1989; Thim, 1989).

Interestingly, pS2 and hSP co-expression is pathologically induced in an EGF/urogastrone-secreting cell lineage in patients with ulcerative gastrointestinal diseases, and a function has been proposed for tissue healing after ulceration (Wright et al., 1990; Rio et al., 1991). Here we characterize two different P-domain peptides from the stomach of *X. laevis*. One represents the *X. laevis* homologue of the pS2 gene product, whereas the second is a novel polypeptide formed by four P-domains arranged in tandem.

**EXPERIMENTAL PROCEDURES**

cDNA Cloning and Northern Analysis—RNA was isolated from the stomach, skin, and brain of adult *X. laevis* (purchased from Dr. W. de Rover, Herpetological Institute, Belgium) by extraction with guanidinium isothiocyanate (Chomczynski and Sacchi, 1987). mRNA was purified using a "Poly(A) Quik kit" (Stratagene). Cyclic thermal amplification of cDNA with Taq polymerase (Perkin-Elmer Cetus Instruments) was according to Frohman et al. (1988). Sequencing of DNA with a "Sequenase kit" (version 2.0, U. S. Biochemicals) using [α-35S]dATP for labeling and computerized analysis have been described previously (Hoffmann, 1988). For homology searches, the FASTA program was applied (Pearson and Lipman, 1988). Northern blots using Hybond-N membrane (Amersham) were performed as reported (Hoffmann and Franz, 1984). As hybridization probes, various restriction fragments were labeled by random priming (Boehringer Mannheim).
Fig. 1. Nucleotide sequence and translation of the xP1 transcript as deduced from cDNA clones pXSP-5'-3.3 (positions 1–258) and pXSP-3'-4.1 (positions 183–483). The potential cleavage site for signal peptidase is indicated by an arrow. The conserved trypsin residue in the P-domain is circled, and the polyadenylation signal and a restriction site are underlined. Also marked are the positions of the synthetic oligonucleotides. The sequence selected for the synthetic peptide (XGP-1) is indicated by a dotted line.

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Fig. 2. Nucleotide sequence and translation of the xP4 transcript as deduced from cDNA clones pXSP-5'-3.27 (positions 1–730) and pXSP-3'-2.1 (positions 304–854). The potential cleavage site for signal peptidase is indicated by an arrow. The conserved trypsin residue in each P-domain is circled, and the polyadenylation signal, restriction sites, and the potential N-glycosylation site are underlined. Also marked are the positions of the synthetic oligonucleotides. The sequence selected for the synthetic peptide (XGP-1) is indicated by a dotted line.

![Fig. 2. Nucleotide sequence and translation of the xP4 transcript as deduced from cDNA clones pXSP-5'-3.27 (positions 1–730) and pXSP-3'-2.1 (positions 304–854). The potential cleavage site for signal peptidase is indicated by an arrow. The conserved trypsin residue in each P-domain is circled, and the polyadenylation signal, restriction sites, and the potential N-glycosylation site are underlined. Also marked are the positions of the synthetic oligonucleotides. The sequence selected for the synthetic peptide (XGP-1) is indicated by a dotted line.](image-url)

Fig. 3. Northern analysis. Hybridization of 20 μg of total RNA from X. laevis skin (lanes a and d), stomach (lanes b and e), and brain (lanes c and f) with the radioactively labeled inserts of cDNA clone pXSP-5'-3.1 (encoding xP1; lanes a–c) or pXSP-3'-2.5 (encoding xP4, similar pXSP-3'-2.1; lanes d–f). As a size marker, a RNA ladder was used (purchased from Bethesda Research Laboratories).

In situ Hybridization Histochemistry—Preparation of X. laevis stomach tissue and conditions for in situ hybridizations were similar to published methods (Königstorfer et al., 1990; Sterrer et al., 1990) except that the RNase A digestion was omitted.

As single-stranded probes, we used 32P- and 3H-labeled in vitro transcripts of various cDNA clones. Prior to use, the in vitro transcripts were digested with RQ1 DNase (Promega).

Production of Antiserum (XGP-1) and Western Analysis—In order to obtain a specific antisera against xP4, the synthetic peptide CYFPDYEDVTIE (XGP-1; kindly provided by C. Hoffmann, Max-Planck-Institut für Psychiatrie) was coupled through the cysteine of the peptide to keyhole limpet hemocyanin with m-maleimidobenzoyl-N-hydroxysuccinimide ester as the coupling reagent (Doolittle, 1986). A rabbit was immunized as previously described (Königstorfer et al., 1989).

Western blots were performed as reported (Königstorfer et al., 1989) using 1:500 dilutions of the antisera. For competitive inhibition of the immunoreactivity, 20 μl of antisera XGP-1 was diluted and preadsorbed with 80 μg of synthetic peptide XGP-1 or XGP-2 (CFYPRATPEY), respectively. As samples, tissue from various organs of X. laevis was boiled with 2% β-mercaptoethanol and centrifuged, and the supernatant was precipitated with acetone.

For analysis of N-linked sugar moieties, the samples were digested with glycopeptidase-F as described (Königstorfer et al., 1989).

RESULTS
cDNA Cloning of xP1 and xP4—Based on the known structure of the four P-domains from FIM-A.1 (Hoffmann, 1988), the oligonucleotide SPL9 d(CCGAATTCGGATCCCTCGA) was constructed. The underlined region represents P-domain sequences. The 3’-sequences were generated after cyclic thermal amplification of oligo(dt)-primed cDNA from X. laevis stomach with SPL9/PCR3’ d(CCCTCGAGATCGAATTC[GTIR]) resulted in products into the EcoRI/XhoI sites of pbScript-2/SK+ (Stratagene), cDNA clones pXSP-5’-4.1 and pXSP-3’-2.1 were obtained.

From the sequences of these two cDNA clones, the complementary oligonucleotides XSPl d(CCCGAATTCGGATCCGAGGAATTC[GTIR]) and XSP3 d(CCCTCGAGATCGAATTC[GTIR]) were designed.

After subcloning the products into the EcoRI/XhoI sites of pbScript-2/SK+ (Stratagene), cDNA clones pXSP-5’-4.1 and pXSP-3’-2.1 were obtained.

For detection of the specific transcripts, XGP-1 and XGP-4, we produced two different enzymes in vitro, 32P-labeled cDNA with PCR5’ d(CCCTCGAGATCGAATTC[GTIR]) and XSP1 d(CCCTCGAGATCGAATTC[GTIR]), respectively, were digested. Then, the 5’-ends were amplified after dC-tailed cDNA with PCR5’/XSP1. Subcloning the products into the EcoRI/XhoI sites of pbScript-2/SK+ resulted in cDNA clones pXSP-5’-3.3 and pXSP-5’-2.1, respectively.

Figs. 1 and 2 represent the full length sequences of the xP1 and xP4 transcripts. They encode different precursors with about 900 nucleotides, whereas the xP4 transcript is comprised of 483 and 854 base pairs without the poly(A) tracks, respectively. The size of the xP1 transcript was determined to be 550 nucleotides, whereas the xP4 transcript comprised about 900 nucleotides. This is in agreement with the lengths obtained by cDNA cloning (483 and 854 base pairs without the poly(A) tracks, respectively). The shadow at about 550 nucleotides in lane d does not originate from xP4 but from...
the preceding hybridization with xP1 due to insufficient stripping of the blot.

In Situ Hybridizations—A very similar expression pattern was obtained for xP1 and xP4. Shown are fundic regions of the stomach (Fig. 4). Both polypeptides are synthesized in surface mucous cells of stomach mucosa. Positive signals were only obtained with probes representing the antisense strands of the xP1 and xP4 transcripts (Fig. 4, A and D), but not with the sense probes (Fig. 4, C and F).

Western Analysis of xP4—In extracts of X. laevis stomach, the antiserum against the C-terminal end of xP4 (XGP-1) recognizes mainly products with $M_r = 25,000$ and about $29,000$ (Fig. 5, lane a) which are shifted after digestion with glycopeptidase-F into a single band with $M_r = 24,000$ (lane b). Immunoreactivity can be blocked by competition with peptide XGP-1 (lane c) but not with the unrelated peptide XGP-2 (lane d). The preimmune serum does not recognize these bands (lane e).

Furthermore, xP4 was also detected in the gastric juice but not in the pancreas, duodenum, or small intestine (data not illustrated).

**DISCUSSION**

Homologies—xP1 and xP4 represent typical P-domain peptides as indicated by comparison with all P-domains identified in X. laevis so far (Fig. 6). The 6 cysteine residues, a single tryptophan, an arginine, and 2 glycine residues are invariant. Generally, the positions of all 6 cysteine residues are highly conserved except for the variable distance between $C_1$ and $C_2$ (6-10 amino acid residues). At the C-terminal end, each P-domain is flanked by at least 1 proline residue. Interestingly, the P-domains identified in X. laevis skin proteins (FIM-A.1, p75k) and those from stomach polypeptides (xP1, xP4) form separate subgroups.

Based on their common structure and their identical expression pattern, but not on particular pronounced homology, xP1 is designated as the obvious X. laevis homologue of pS2. So far, xP2 has only been detected in primates, and previous attempts failed to characterize a homologous sequence in other vertebrates (Rio et al., 1988a). In contrast, xP4 represents a novel polypeptide with four P-domains arranged in
tandem. This would be equivalent to a duplicated form of the spasmodic polypeptide (PSP, mSP, hSP). Currently, it is not known if an exact PSP homologue exists in X. laevis.

A homology search showed also an interesting relationship (21% identity in 194 aminos acid overlap) between xP4 and the heavy chain of human hepatocyte growth factor family (Miyazawa et al., 1989; Nakamura et al., 1989; Rubin et al., 1991) since this polypeptide mainly consists of four kringle domains (data not illustrated). 

Biosynthesis—Hydropathy profiles (Kyte and Doolittle, 1982) revealed that both deduced precursors (Figs. 1 and 2) contain hydrophobic sequences at their N-terminal ends as typical of secretory proteins (data not illustrated). Possible cleavage sites for signal peptidase according to von Heijne (1983) are indicated with arrows. The N-terminal glutamine residue from xP1 is probably enzymatically cyclized into a pyroglutamic acid residue (pGlu; Busby et al., 1987; Fischer and Spiess, 1987) similar to a variety of secretory proteins including PSP (Rose et al., 1989). Whether the hypothetical N-terminal glutamic acid residue in xP4 is also a target for cyclization remains to be determined. Interestingly, the pS2 polypeptide begins at its N-terminal end with a glutamic acid residue which is probably cyclized to pGlu in the stomach but not in CDF-7 cells (Rio et al., 1988b; Mori et al., 1988) and partial cyclization of glutamic acid residues has also been reported for α-lipoprotein and joining peptide (Bateman et al., 1990). The potential N-glycosylation site in xP4 is obviously modified leading to different glycosylation variants (M, 29,000 and 26,000; Fig. 5). Further potential processing sites are five pairs of basic residues in xP4. Since the antibody (XGP-1) against the predicted C-terminal end of xP4 recognizes a deglycosylated protein with M, 24,000 (Fig. 5) as predicted from cDNA cloning, cleavage can be excluded. This is analogous to uncleaved basic pairs in FIM-A1 (Hauser et al., 1990) and PSP (Rose et al., 1989).

Expression—xP1 and xP4 are secretory products of the stomach mucosa (Fig. 4). Interestingly, there is a steep gradient in expression with a decrease from fundic regions to the antrum. Due to their similar expression pattern and their homology with pS2 and hSP, comparable physiological function can be expected for xP1 and xP4. Among these, the most challenging is certainly a potential growth factor activity. This hypothesis would also be in line with the relationship between xP4 and human hepatocyte growth factor and with the general appearance of P-domains, which is reminiscent of EGF/urogastrone and EGF-like repeats. Such a growth-promoting activity could account for the general high turnover rate of gastric mucosal cells and especially for regeneration after injury. Preceding this process, for preliminary very rapid repair, gastric mucosa is capable of restituting first its epithelial integrity, even in vitro after nearly complete destruction of the surface epithelium, e.g. with hyperosmolar sodium chloride (Silen, 1987).

For further understanding of the molecular function of the novel polypeptide xP4, it will be essential to investigate the regulation of this gene and to search for xP4 receptors. However, it remains to be determined if a homologue of xP4 exists in mammals.

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P-Domain Peptides from X. laevis