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An Integumentary Mucin (FIM-B.1) from *Xenopus laevis* Homologous with von Willebrand Factor†

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ABSTRACT: We present a new protein from *X. laevis* skin termed "frog integumentary mucin B.1" (FIM-B.1) with a general structure similar to FIM-A.1 (formerly "spasmolysin"). The central region consisting of tandem repeats of 11 amino acid residues is probably a target for extensive O-glycosylation, whereas the C-terminal cysteine-rich domain shows pronounced homology with the C1-C2 domains and the C-terminal end of von Willebrand factor. Furthermore, we describe homology with antistasin, an anticoagulant peptide from a leech. We also discuss some implications concerning the evolutionary origin of von Willebrand factor. In situ hybridization studies revealed the expression of FIM-B.1 exclusively in mucous glands of the skin. This is comparable with FIM-A.1 but is in contrast to all other physiologically active peptides, which are synthesized in granular glands.

In the past, secretions from frog skin turned out to be the source for biogenic amines and a variety of different physiologically active peptides (Erspamer, 1971), which probably act in defense mechanisms. Due to the embryonic relationships of the tissues (Alpert et al., 1988), many of these peptides have counterparts in the nervous system as well as in the gastrointestinal tract (Erspamer & Melchiorri, 1980; Pearse, 1979). *Xenopus laevis* skin contains two different types of glands—granular (or poison) and mucous glands (Engelmann, 1872). All of the active polypeptides investigated so far are

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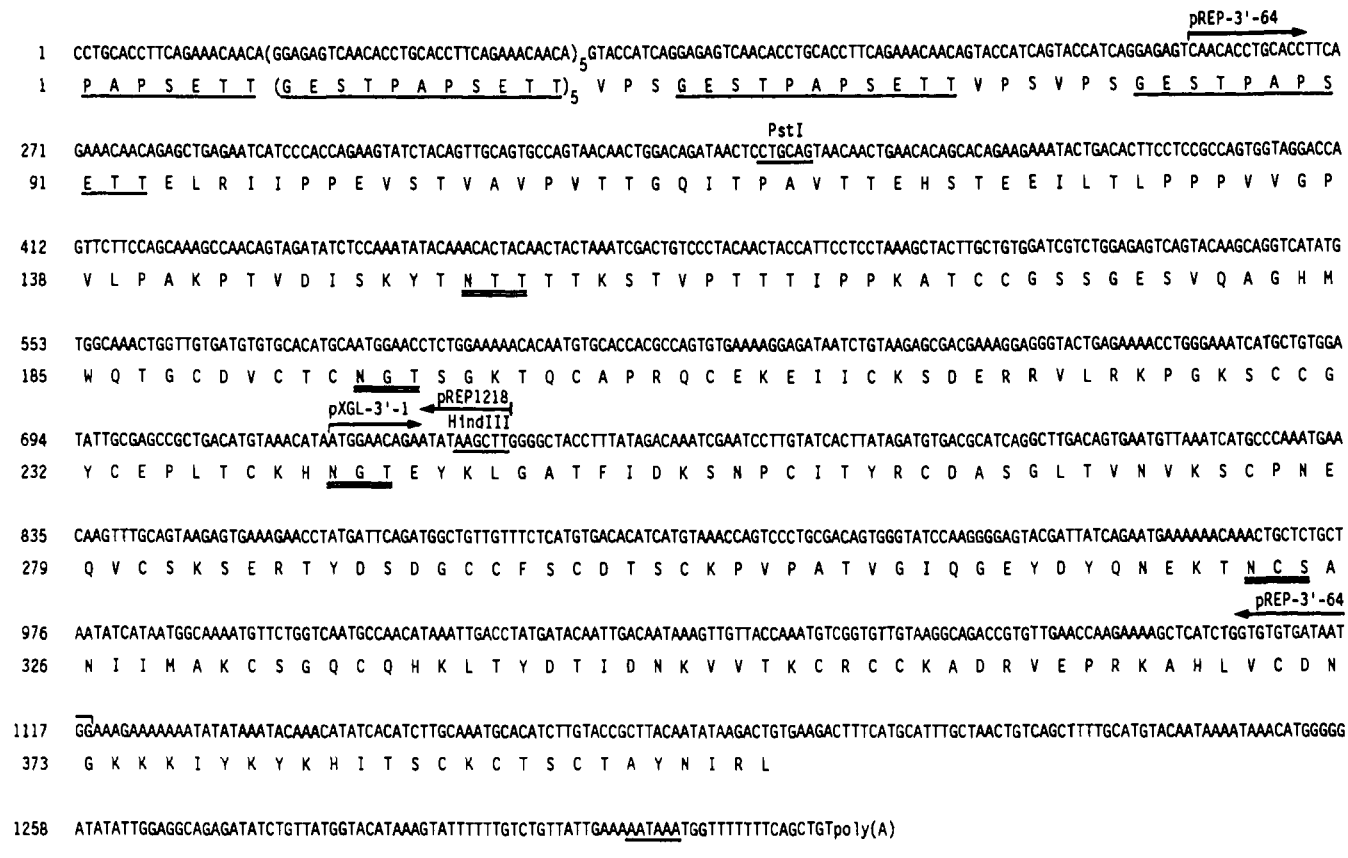

FIGURE 1: Nucleotide sequence of the 3'-part of the FIM-B.1 transcript and translation in the open reading frame, as derived from cDNA clones pREP1218, pREP-3'-64, and pXGL-3'-1. Restriction sites, the polyadenylation signal (Proudfoot & Brownlee, 1976), repetitive elements, and potential N-glycosylation sites are underlined.

synthesized by granular glands. After stimulation, extrusion occurs by a holocrine mechanism (Dockray & Hopkins, 1975; Bennet et al., 1981; Flucher et al., 1986).

In contrast, there is only a single secretory protein known, which is expressed and stored exclusively in mucous glands of *X. laevis* skin (Hauser et al., 1990). This "frog integumentary mucin A.1" [FIM-A.1; originally proposed as "spasmolysin"; see Hoffmann (1988)] consists of a central threonine–proline-rich repetitive domain and terminal cysteine-rich domains, which were termed recently "P-domains" (Tomasetto et al., 1990) and are found otherwise in the pancreatic spasmolytic polypeptide (PSP), the pS2 gene product from human antrum, the sucrase–isomaltase complex, and α-glucosidase (Hoffmann, 1988; Tomasetto et al., 1990). As with human intestinal mucins (Gum et al., 1989), FIM-A.1 is extensively O-glycosylated ($\geq$50% carbohydrate moiety) at its central type-A repeats resulting in a MW of about 130K (Hauser et al., 1990).

During the course of cloning the FIM-A.1 precursors, a new homologous repetitive element (rep-33) with the predicted amino acid sequence GESTPAPSETT was discovered (Hoffmann, 1988), which is now classified as a type-B repeat (Hauser et al., 1990). Here, we characterize and study the expression of a not-yet described integumentary mucin from *X. laevis* designated FIM-B.1 containing this type-B repeat followed by a new cysteine-rich C-terminal domain.

Materials and Methods

cDNA Cloning. Construction of an oligo(dT)-primed cDNA library from *X. laevis* skin has been described (Hoffmann, 1988). Screening of this cDNA library with the synthetic oligonucleotide REP1 d(AAGGTGCAGGTGTTGACT) (hybridization temperature 49 °C) was according to Singer-Sam et al. (1983). The full 3'-sequence was obtained after cyclic thermal amplification with Taq-Polymerase (Perkin-Elmer Cetus) as described (Frohman et al., 1988) and subcloning the products in a pBluescript II/SK$^-$ vector (Stratagene).

Sequencing of DNA, sequence analysis, and protein homology searches (FASTP program) have been described previously (Hoffmann, 1988).

In Situ Hybridization. Pieces of *X. laevis* skin were embedded in Tissue-Tek (Miles), cut with a cryostat into 15-μm sections, mounted on gelatin-treated glass slides, and fixed with 4% paraformaldehyde for 15 min at room temperature. After acetylation (Angerer et al., 1987) the conditions for prehybridization, hybridization, washing, RNAse A digestion, and autoradiography were according to Bandtlow et al. (1987). Before they were covered with Entellan (Merck), the sections were histologically stained with hematoxylin–eosin (Romeis, 1968).

As FIM-B.1-specific single-stranded RNA probes we used ^{32}P- or ^{3}H-labeled in vitro transcripts of the PstI fragment representing the nonrepetitive part of pREP1218 subcloned in pBluescript/SK$^-$ (Stratagene). Alternatively, the synthetic oligonucleotide REP1 after tailing with ^{32}P-labeled dATP (Watson et al., 1987) was hybridized. In this case, RNAse A digestion following hybridization and washing was omitted.

Results

Molecular Characterization. Screening of an oligo(dT)-primed cDNA library from *X. laevis* skin (Hoffmann, 1988) with the synthetic oligonucleotide REP1 d-(AAGGTGCAGGTGTTGACT), which is complementary to the underlined part of the rep-33 sequence d-(GGAGAGTCAACACCTGCACCTTCAGAAACAACA)

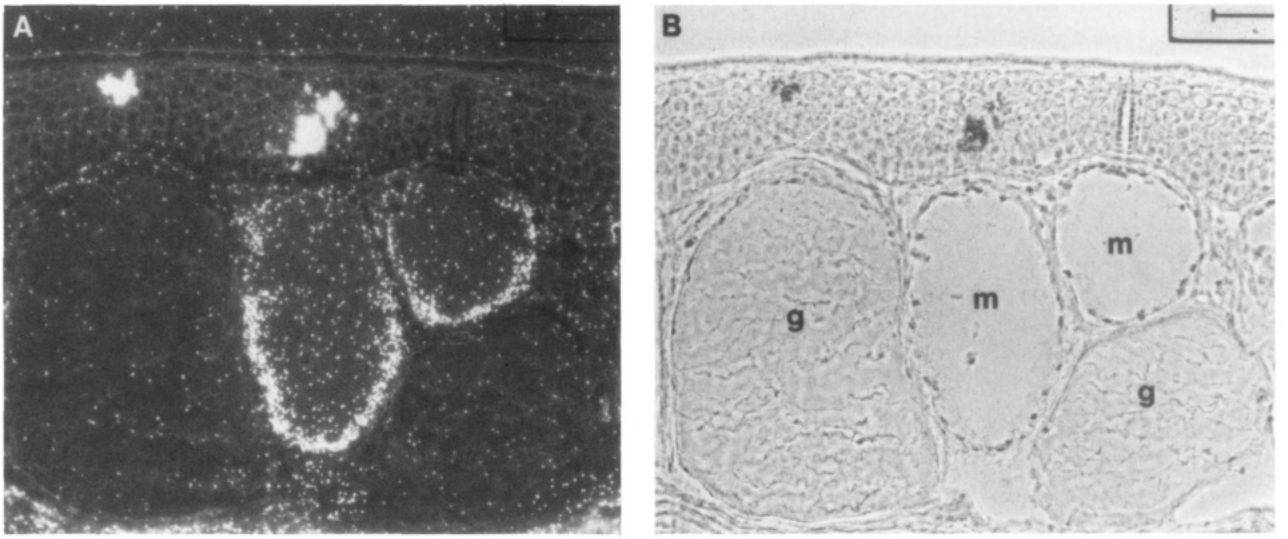

FIGURE 2: In situ hybridization of ventral *X. laevis* skin. As a probe we used ^{3}H-labeled single-stranded cRNA (*Pst*I fragment from pREP1218). Exposure time for autoradiography, 8 weeks. (A) Dark-field illumination representing FIM-B.1 mRNA solely in mucous gland cells. (B) Phase-contrast picture. Scale bar, 50 μm; g, granular gland; m, mucous gland.

(Hoffmann, 1988), resulted in the clone pREP1218. This clone encodes part of the FIM-B.1 precursor (positions 1–741 in Figure 1).

The sequence of the complete 3′-end of the FIM-B.1 mRNA was obtained after cyclic thermal amplification. As primers we used the following combinations of oligonucleotides, resulting in cDNA clones pREP-3′-64 or pXGL-3′-1, respectively: REP3 d(CCGAATTCTCGAGGATCCAACACCTGCACCTTCA)/PCR3′ d(CCCTCGAGGATCCGAATTC[T]$_{18}$) or XGL1 d-(CCGAATTCTCGAGAGATCTATGGAACAGAATATAAGC)/PCR3′. Underlined regions represent FIM-B.1 sequences. cDNA clone pREP-3′-64 results from priming with PCR3′ at an internal A-rich region whereas pXGL-3′-1 was primed directly at the poly(A) tail.

Figure 1 represents the 3′-part of a novel mRNA from *X. laevis* skin, encoding part of the FIM-B.1 precursor starting with multiple repetitive elements (rep-33). Translation into the single open reading frame clearly designates two characteristic regions: a repetitive domain, rich in threonine and proline residues, and a cysteine-rich C-terminal domain starting at position 171.

At present, the length of the entire mRNA is not exactly known. But we expect a rather large mRNA since repeated Northern analysis with nonrepetitive probes as well as primer extension experiments yielded consistently a smear up to 10 kb (data not illustrated). A similar result was obtained by using a rep-33 probe (Hoffmann, 1988).

Expression Studies. As shown in Figure 2, FIM-B.1 mRNA is exclusively synthesized in mucous glands from *X. laevis* skin and not at all in granular glands. In situ hybridization revealed positive signals only with an antisense cRNA probe and an oligonucleotide probe (REP1) representing the antisense strand. With an in vitro transcript representing the sense strand of FIM-B.1 mRNA, no signals could be obtained (data not illustrated).

Discussion

Homologies. The deduced amino acid sequence of FIM-B.1 (see Figure 1) exhibits remarkable similarities with that of the FIM-A.1 precursor (Hoffmann, 1988)—the general structure of both follows the same scheme. Both contain a homologous threonine–proline-rich repetitive domain as well as a cysteine-rich C-terminal domain. Therefore, we predict that the repetitive domain in FIM-B.1 is the target for extensive O-glycosylation as is the case for FIM-A.1 (Hauser et al., 1990). Similar highly O-glycosylated repetitive sequences have also been identified in a human polymorphic epithelial mucin (Gendler et al., 1988).

Other processing signals recognized are four potential N-glycosylation sites within the cysteine-rich domain (Figure 1). In particular, this region should be responsible for the physiological activity besides the character as a mucin protecting the delicate skin.

A first homology search revealed characteristic features shared with two other proteins, i.e., human von Willebrand factor (vWF), a multifunctional adhesive glycoprotein essential for hemostasis (Sadler et al., 1985; Shelton-Inloes et al., 1986; Titani et al., 1986; Verweij et al., 1986), and antistasin, an anticoagulant and antimetastic peptide from a Mexican leech (Nutt et al., 1988), which binds to a specific sulfatide (Holt et al., 1989).

As outlined in Figure 3A, homology in the frog sequence is confined to the cysteine-rich domain (dotted area in FIM). Nearly all cysteine residues in FIM-B.1 appear in equivalent positions in vWF, indicating a very similar tertiary structure (see Figure 3B). Homologous positions in vWF are at the C-terminal end of the molecule starting with the C1 domain. But in contrast to vWF, where the homologous C1 and C2 domains arose by duplication, in FIM-B.1 this duplication did not occur. In general, the frog sequence is more related to the C1 than the C2 domain; especially in C1, one can detect the extremely conserved region (WXXGCDVCTC). Another conserved region (43% identity) is close to the C-terminal end (Cys^{1987} to Gly^{1228} in vWF; Titani et al., 1986). On the other hand, the major difference between FIM-B.1 and vWF is certainly the cell attachment site Arg-Gly-Asp (Ruoslahti & Pierschbacher, 1985), which is present in vWF only (underlined in Figure 3B). Also very interesting is a comparison between FIM-B.1 and antistasin (Figure 3B). In particular, six cysteine residues in the central part of antistasin (positions 48, 51, 53, 62, 67, and 73; Dunwiddie et al., 1989) are highly conserved. On the basis of the repetitive internal structure of antistasin, alignment with homologous regions revealed that this leech peptide could possibly be the ancestor of the C1–C2 domains in vWF (see Figure 3A). So, hypothetically, starting

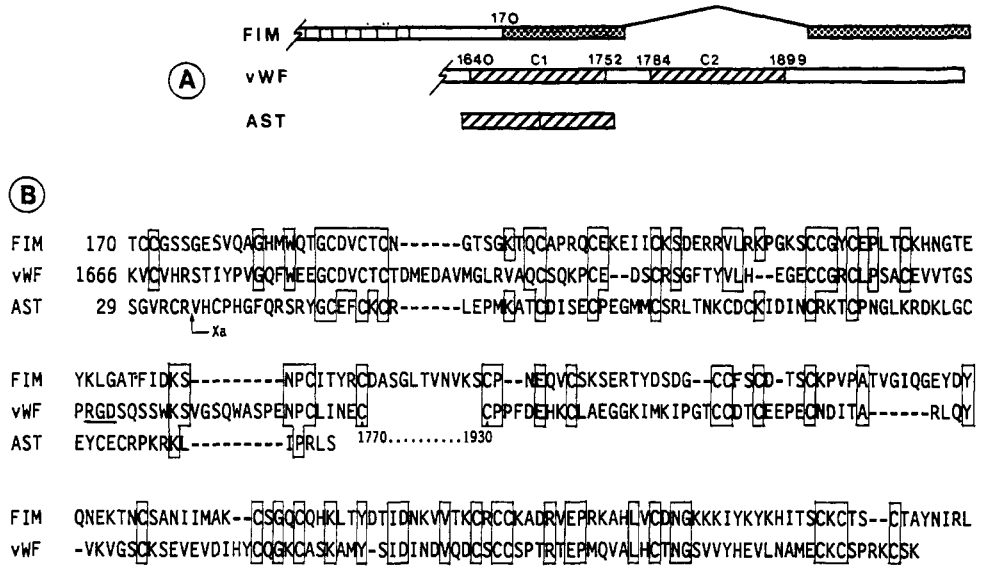

FIGURE 3: Homology of FIM-B.1/vWF/antistasin. (A) Schematic representation of the homologous regions from FIM-B.1, vWF [numbering according to Titani et al. (1986)], and antistasin (AST). The cysteine-rich region in FIM-B.1 is dotted, vertical bars indicate type-B repeats (elongation by insertion of VPS sequences is represented by dots). The C1–C2 domains of vWF and AST are hatched. (B) Comparison of the amino acid sequences from FIM-B.1 (FIM; starting with position 170), vWF (starting with position 1666; Titani et al., 1986), and AST (starting with position 29; Dunwiddie et al., 1989). Gaps are introduced to maximize homology; identical amino acid residues in vWF and AST when compared with FIM are enclosed in boxes. In vWF 25% of all positions are identical; about half of them are represented by cysteine residues. The RGD sequence in vWF is underlined. Also shown is the cleavage site for factor Xa in AST.

with two nearly identical repetitive units in antistasin, changes appear to have occurred mainly at both ends, creating a froglike sequence, which later on is duplicated to form the C1–C2 domains. Taken together, FIM-B.1 from *X. laevis* skin as predicted from cDNA cloning has counterparts in a peptide from a leech as well as in vWF synthesized in endothelial cells and megakaryocytes. Furthermore, similar as described for vWF (Hunt & Barker, 1987), we also could detect sequence similarities between FIM-B.1 (positions 172–235) and a cysteine-rich domain in procollagen and thrombospondin (data not illustrated).

Possible Function of FIM-B.1. Antistasin has been reported to be a potent inhibitor of activated factor Xa (Tuszynski et al., 1987) and recently it has been shown that this is due to a cleavage of antistasin by factor Xa after Arg^{34} (Dunwiddie et al., 1989). On the other hand, the C-terminal end of vWF is essential for dimerization via disulfide bonds and the RGD sequence within the C1 domain is probably involved in binding the platelet receptor GPIIb-IIIa [for a review see Sadler and Davie (1987) and Titani and Walsh (1988)]. However, on the basis of the homologies (Figure 3), no direct molecular mechanism is evident for a physiological role for FIM-B.1. Since both homologous sequences are either involved or interfere with the blood coagulation process, we tentatively assume some anticoagulant activity for the mucin from *X. laevis* skin. Interestingly, such an activity is indeed present in *X. laevis* skin secretions (K. Rübsamen, personal communication). The fact that FIM-B.1 is solely expressed in mucous glands and not at all in granular glands presents another striking similarity with FIM-A.1 (Hauser et al., 1990) and is also in line with the predicted mucinlike character of the protein. Furthermore, this could lead to the assumption that both FIMs described so far could fulfill their yet-unknown biological function together as parts of the same multicomponent system on the surface of *X. laevis* skin—maybe for defense against microbial infections (Hauser et al., 1990). In this context it is worth mentioning that we have recently detected a mucin containing a motif typical of many proteins of the complement system (Probst and Hoffmann, unpublished experiments).

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Registry No. von Willebrand factor, 109319-16-6; antistasin, 110119-38-5.

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Calcium Binding in α-Amylases: An X-ray Diffraction Study at 2.1-Å Resolution of Two Enzymes from *Aspergillus*[†,‡]

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ABSTRACT: X-ray diffraction analysis (at 2.1-Å resolution) of an acid α-amylase from *Aspergillus niger* allowed a detailed description of the stereochemistry of the calcium-binding sites. The primary site (which is essential in maintaining proper folding around the active site) contains a tightly bound Ca^{2+} with an unusually high number of eight ligands (Oδ1 and Oδ2 of Asp175, Oδ of Asn121, main-chain carbonyl oxygens of Glu162 and Glu210, and three water molecules). A secondary binding site was identified at the bottom of the substrate binding cleft; it involves the residues presumed to play a catalytic role (Asp206 and Glu230). This explains the inhibitory effect of calcium observed at higher concentrations. Neutral *Aspergillus oryzae* (TAKA) α-amylase was also refined in a new crystal at 2.1-Å resolution. The structure of this homologous (over 80%) enzyme and additional kinetic studies support all the structural conclusions regarding both calcium-binding sites.

Calcium (Ca^{2+}) ions are bound by a wide variety of intra- and extracellular proteins, including enzymes and structural, transport, and trigger proteins. Their binding affinities are commonly in the range 10^5-10^8 M^{-1}. No unifying structural basis for this broad range has been established.

Ca^{2+} binding is an important biochemical phenomenon with many widespread consequences. One of the reasons why Ca^{2+} has been so extensively utilized by nature may be the variability of its coordination. In spite of its preference toward octahedral coordination, up to nine ligands can be found in crystalline complexes involving calcium–carboxylate and calcium–water interactions (Einspahr & Bugg, 1980, 1981); this variability makes the regulation of the binding affinity an easier task. A complete structural description of these mechanisms is necessary to understand the ways in which proteins' activities can be modulated by Ca^{2+}. It should also be noted that purely structural Ca^{2+} sites contribute substantially to the stability of the proteins. Recent advances in protein engineering have often been directed at obtaining more stable enzymes suitable for industrial use under demanding conditions. The under-

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