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Cryptic Origin of SPAI, a Plasma Protein with a Transglutaminase Substrate Domain and the WAP Motif, Revealed by *in Situ* Hybridization and Immunohistochemistry*

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SPAI, originally isolated as a sodium/potassium-ATPase inhibitor and now considered to be a proteinase inhibitor of unknown specificity based on its similarity to elafin (an elastase inhibitor), is a new type of plasma protein that has a transglutaminase substrate domain, which serves as an anchoring sequence to be covalently cross-linked at target sites. To determine the source of SPAI, we carried out *in situ* hybridization and immunohistochemistry using an antisense cRNA probe and an antiserum against recombinant SPAI, respectively. Since previous RNase protection analysis had indicated that SPAI mRNA is almost exclusively expressed in the porcine small intestine, we used its frozen sections for the staining. The lower crypt was decorated with both the cRNA probe and antiserum, indicating that SPAI is synthesized and secreted by the enteroendocrine cells located near the crypt base. The native form of SPAI was also characterized by Western blotting. This result together with the previous biochemical and molecular biological characterizations may set the stage for identifying the physiological roles of the conceptually very interesting protein SPAI.

The protein motif WAP[1] (1) is characterized by 8 conserved cysteine residues that form 4 disulfide bonds, a structure called "four-disulfide core," and is found in a wide variety of proteins including whey proteins, proteinase inhibitors, neurophysins, plant agglutinin, adhesion molecules, scorpion toxins, bactericidal peptides, pollen proteins, and SPAI (Ref. 2, and references cited therein). SPAI is the latest member of this WAP protein superfamily. The name WAP derives from the first well characterized member whey acidic protein, which is the most abundant protein in rodent milk (3).

SPAI was first isolated from the porcine duodenum as a 61-amino acid polypeptide with a Na^+,K^+-ATPase inhibitory activity (4). Its true physiological function, however, remains to be established since its IC_{50} for renal Na^+,K^+-ATPase is relatively high, around 10^{-6} M (5). Cloning and expression of SPAI cDNA established that SPAI has an N-terminal extension of 105 amino acid residues that serves as a transglutaminase substrate domain (2). In this respect, SPAI resembles elafin, an elastase inhibitor, which we have previously characterized and shown to be composed of two domains: a transglutaminase substrate domain and the WAP motif (6, 7). SPAI and elafin, therefore, represent a conceptually new type of protein that has an anchoring sequence which helps to confine the active WAP motif region at its site of action and constitutes a subfamily of the WAP protein superfamily. Structural features of SPAI and elafin are schematically shown in Fig. 1. In the case of elafin, its transglutaminase-mediated *in vivo* cross-linking to extracellular matrix proteins through the transglutaminase substrate domain has been demonstrated in the trachea (7) and skin (8, 9). Transglutaminase is an enzyme catalyzing the covalent cross-linking of specific proteins by the formation of stable isopeptide bonds between Gln and Lys side chains (10–14). The transglutaminase substrate domains of SPAI and elafin consist of a repetitive sequence rich in Gln and Lys residues.

An interesting feature of SPAI is its presence in the circulation (2). Based on its circulatory nature, we hypothesized that SPAI may accumulate covalently at the site of wound and inflammation, where plasma and tissue transglutaminases are activated, and may serve to stimulate wound healing and to limit inflammatory reactions. One of the important issues yet to be addressed is where the circulating SPAI comes from. To answer this question, we performed, in the present study, *in situ* hybridization and immunohistochemistry and showed that the crypt of the intestine is the most likely site of synthesis and secretion of SPAI. We also confirmed that native SPAI is a 166-amino acid residue protein, as expected from the cDNA sequence analysis. To distinguish the native form from the originally isolated shorter form (61 amino acid residues) lacking the transglutaminase substrate domain, we use, in this communication, the term SPAI (WAP-2) for the native form.

EXPERIMENTAL PROCEDURES

Materials—Porcine small intestine was obtained from the Hachioji abattoir sanitation inspection station, Tokyo, Japan. Restriction enzymes were obtained from Toyobo, Osaka, Japan; DNA ligation kit (version 2) and *Taq* polymerase were from Takara, Otsu, Japan; Vectabond reagent was from Vector Laboratories, Inc., Burlingame, CA.

Preparation of Template DNA—Using the following set of primers, an appropriate region of a full-length SPAI (WAP-2) cDNA was amplified, subcloned into the pBluescript II vector, and used as a template for cRNA synthesis: 5′-ACAGTCATAATCTCTCCAACAC-3′ (nucleotides 483–504, Ref. 2) and 5′-AGTCCCAGGTCGATTCCTTCTC-3′ (nucleotides 399–420, Ref. 2). The polymerase chain reaction was performed using 1 μg of *Eco*RI-linearized plasmid DNA harboring a complete cDNA of SPAI (WAP-2), 100 pmol of each primer, and the following cycling conditions: 1 min at 94 °C for denaturation, 1 min at 55 °C for primer annealing, and 2 min at 72 °C for elongation. This cycle was

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[1] The abbreviations used are: WAP, a protein motif first characterized in whey acidic protein; SPAI, a WAP motif protein with a weak sodium/potassium ATPase inhibitory activity that is generated by acid hydrolysis from SPAI (WAP-2); SPAI (WAP-2), second member of the subfamily of the WAP protein superfamily, which has a transglutaminase substrate domain; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis.

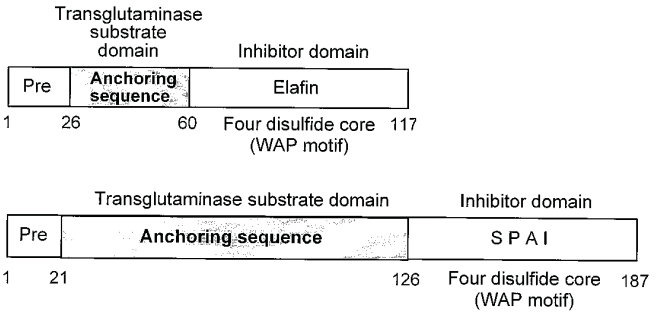

FIG. 1. **Domain structure of SPAI (WAP-2).** SPAI (WAP-2) belongs to the WAP protein superfamily whose members have a characteristic "four-disulfide core" structure or the WAP motif. Among the members, SPAI and elafin, an elastase inhibitor (23), constitute a subfamily having in their N terminus a unique additional sequence that serves as a transglutaminase substrate domain and helps to anchor the WAP domain covalently at its site of action. The transglutaminase substrate domain of SPAI (WAP-2) consists of 16 repeats of semiconserved 6 amino acid residues (GQDPVK) and is rich in Lys and Gln (2); the transglutaminase substrate domain of elafin consists of 5 hexapeptide repeats (6). The *numbers* indicate the positions of amino acids residues. *Pre*, presequence; *SPAI*, $SPAI^{127–187}$; SPAI (WAP-2) corresponds to $SPAI^{22–187}$.

repeated 35 times with a final extension at 72 °C for 10 min. Polymerase chain reaction products (106 base pairs) were then separated on a 1% agarose gel, isolated, phosphorylated, and ligated into the *Eco*RV site of pBluescript II SK^-.

Preparation of Digoxigenin-labeled cRNA Probes—Antisense and sense RNA probes were transcribed from the template plasmid linearized by *Eco*RI and *Hin*dIII in the presence of digoxigenin-UTP (Boehringer Mannheim) with T3 and T7 RNA polymerases (Stratagene) according to the manufacturer's protocol.

In Situ Hybridization Histochemistry—Fresh porcine intestine was cut into small pieces, fixed for several hours at room temperature in 4% paraformaldehyde in phosphate-buffered saline (PBS; 10 mM phosphate, pH 7.4, 145 mM NaCl), immersed in 30% sucrose in PBS for cryopreservation, embedded in Tissue Tek O.C.T. compound (Miles), and snap frozen. Six-μm cryosections were cut and mounted on Vectabond-treated glass slides and baked at 56 °C for 30 min and then at 65 °C for 1 h to block endogenous alkaline phosphatase. The sections were then processed by standard techniques (15), and hybridization signals were visualized with sheep polyclonal anti-digoxigenin Fab fragments conjugated to alkaline phosphatase (Boehringer Mannheim) using 4-nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate as substrates for color reactions in alkaline phosphatase buffer (0.1 M Tris-HCl, pH 9.0, 0.1 M NaCl, 50 mM $MgCl_2$). Nonspecific hybridization was determined by parallel incubation of adjacent sections with sense riboprobe.

Antibody Production—Polyclonal antibodies to SPAI (WAP-2) were raised by immunizing New Zealand White rabbits with 100 μg of recombinant SPAI (WAP-2) expressed in *Escherichia coli* and purified as described previously (2). Immunization was boosted by injecting biweekly 50 μg of antigen three times.

Immunohistochemistry—Sections were prepared as described above, baked at 56 °C, immersed twice in PBS for 10 min, preblocked with 5% goat serum in PBS for 1 h at room temperature, and incubated with primary antisera (1:5000) in PBS containing 5% goat serum at 4 °C overnight. They were washed with PBS and were incubated with the secondary antibody, goat alkaline phosphatase-conjugated anti-rabbit IgG antibody (Bio-Rad, 1:5000), at 4 °C for 4 h. After washing with PBS, the sections were immersed in alkaline phosphatase buffer and incubated with 4-nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate.

Western Blotting—Epithelium of porcine small intestine was scraped with a blade and homogenized in 2 volumes of PBS at 4 °C. The homogenate was centrifuged at 500 × *g* for 2 min at 4 °C to remove nuclei and large debris, and the supernatant was further homogenized in SDS-PAGE sample buffer (10 mM phosphate, 2% SDS, 20 mM dithiothreitol, 4% glycerol, pH 6.8) using a 1-ml Teflon homogenizer and subjected to Western blot analysis as described previously (7) with the following modifications. Working dilutions of the primary and secondary antisera were 1:5000. SPAI (WAP-2), used as a reference sample, was prepared as described previously (2).

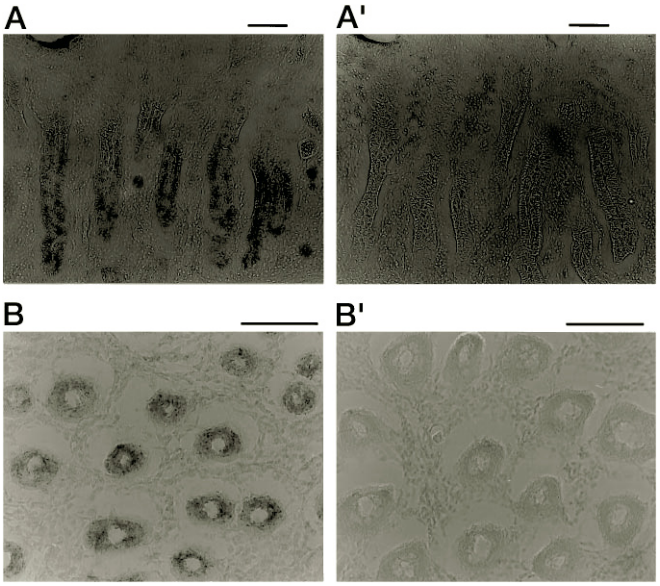

FIG. 2. **Histochemical analysis of SPAI (WAP-2) mRNA localization in porcine small intestine by *in situ* hybridization.** Pieces of formaldehyde-fixed porcine small intestine were cut parallel (*A* and *A′*) or perpendicular (*B* and *B′*) to the crypt-villus axis, hybridized with a digoxigenin-labeled antisense (*A* and *B*) and the control sense (*A′* and *B′*) probe. Signals were developed with an anti-digoxigenin antibody conjugated to alkaline phosphatase using 4-nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate as chromogen. Original magnification, × 200 (*A* and *A′*) and × 400 (*B* and *B′*). *Bars* correspond to 5 μm.

RESULTS

In Situ Hybridization and Immunohistochemistry—Previously we demonstrated by Northern blot and RNase protection analyses that SPAI (WAP-2) is almost exclusively expressed in the porcine intestine, especially in the small intestine (2, 16). To localize more precisely the cellular sites of expression of SPAI (WAP-2) mRNA, we performed *in situ* hybridization histochemistry on porcine small intestine sections with digoxigenin-labeled riboprobes. Prominent hybridization signals were observed in the flask-shaped mucosal invagination known as the crypt of Lieberkühn (Fig. 2, *A* and *B*). The signals, however, were not distributed evenly throughout the crypts; they were confined to the cells in a lower part of the crypt. No detectable expression of the message was found in the remaining parts of the section. The control sense probe did not show any specific staining (Fig. 2, *A′* and *B′*).

The expression pattern was substantiated by immunohistochemical staining (Fig. 3). The antiserum directed against recombinant SPAI (WAP-2) specifically stained the crypt cells of the porcine small intestine (Fig. 3, *A* and *B*). The preimmune serum and the control serum preabsorbed with the antigen did not produce any staining (Fig. 3, *A′* and *B′*).

Native Form of SPAI (WAP-2) in Intestine—SPAI (WAP-2) is a secretory protein whose nascent form has a presequence on its N terminus. Knowing the fate of SPAI (WAP-2) after its synthesis in and secretion from the crypt cells is important for elucidating its physiological roles. To address this point, we determined the size of the native form of SPAI (WAP-2) in the intestine; the rationale of this approach is that if SPAI (WAP-2) is cross-linked, like elafin (WAP-1) in the trachea (7), to the extracellular matrix proteins through its transglutaminase substrate domain, it should be detected as higher molecular weight species, or alternatively if it is the source of plasma SPAI (WAP-2), it should be the same size as the circulating form (~26 kDa, estimated by SDS-PAGE; Ref. 2).

Surface areas, roughly corresponding to the mucosal epithe-

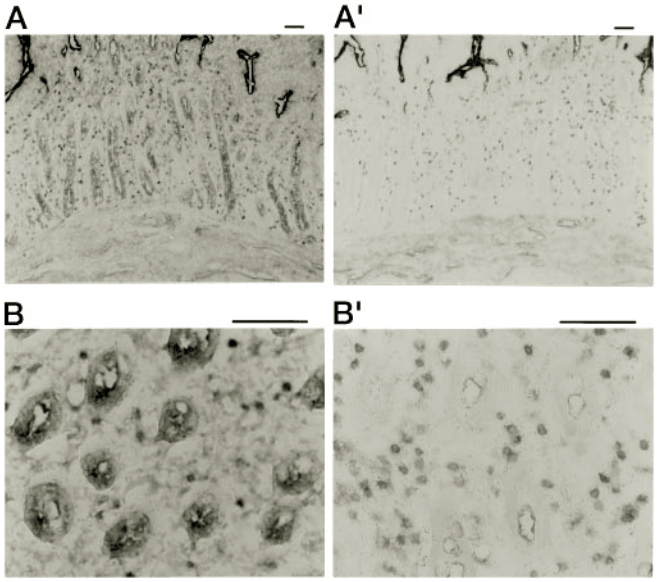

FIG. 3. **Immunohistochemical localization of SPAI (WAP-2) in porcine small intestine.** Immunostaining was performed as described under "Experimental Procedures." *A*, transverse section stained with anti-SPAI (WAP-2) antiserum; *A′*, transverse section stained with preimmune serum. *B*, longitudinal section stained with anti-SPAI (WAP-2) antiserum; *B′*, longitudinal section stained with preimmune serum. Villus cells or columnar absorptive enterocytes are nonspecifically stained both with the antiserum and preimmune serum due to very strong intrinsic alkaline phosphatase activities; the lack of specific staining in the villus was confirmed by replacing the alkaline phosphatase-labeled secondary antibody with tetramethylrhodamine isothiocyanate (a fluorescent dye)-conjugated secondary antibody (data not shown). Original magnification, × 100 (*A* and *A′*) and × 400 (*B* and *B′*). *Bars* correspond to 5 μm.

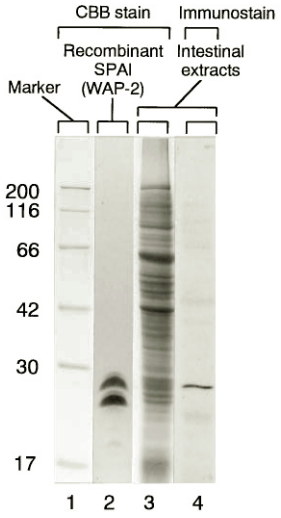

FIG. 4. **Western blot analysis of the SPAI (WAP-2) protein in intestinal epithelium.** Porcine small intestinal epithelium extracts were boiled for 10 min in SDS sample buffer containing 2-mercaptoethanol and electrophoresed on 12% polyacrylamide gel. After separation, the proteins were either stained with Coomassie Brilliant Blue R-250 (*CBB, lane 3*) or electroblotted to nitrocellulose membrane and probed with antiserum against recombinant SPAI (WAP-2) (*lane 4*). *Lane 1*, size markers; *lane 2*, purified authentic recombinant SPAI (WAP-2). The lower band of closely spaced doublet (24/26 kDa) seen in purified authentic SPAI (WAP-2) (*lane 2*) is a proteolytic product of the native form (upper band).

lium, of the porcine intestine containing the crypt/villus units were removed, homogenized in the SDS-PAGE sample buffer, and analyzed by Western blotting using an antiserum directed against recombinant porcine SPAI (WAP-2). Only a single protein band of the same size as the authentic recombinant SPAI (WAP-2) was recognized by the antiserum among a large number of proteins contained in the intestinal extracts (Fig. 4). The absence of higher molecular weight immunoreactive bands indicates that SPAI (WAP-2) remains unconjugated. This fact strongly suggests that the crypt is the major source of plasma SPAI (WAP-2). The simple pattern obtained by Western blot analysis also demonstrates the monospecificity of the antiserum.

DISCUSSION

In the present study, we demonstrated that SPAI (WAP-2) is produced in the crypt cells in the porcine small intestine. The anatomic and functional unit of the mammalian small intestinal epithelium is the crypt of Lieberkühn and its associated villus, which contains several types of cells including 1) columnar absorptive enterocytes, 2) mucous goblet cells, 3) enteroendocrine cells known to secrete a variety of gastrointestinal hormones (17), and 4) Paneth cells at the base of the crypt (18, 19). These cells are considered to arise from multipotent stem cells located near the base of each crypt and differentiate as they slowly migrate along the crypt-villus axis (20, 21). In the case of the porcine small intestine, however, Paneth cells are usually not present. The SPAI (WAP-2) mRNA-positive cells located in the lower crypt (Fig. 2*A*) may, therefore, be enteroendocrine cells in their early stages of differentiation. This localization appears to be quite consistent with the circulatory nature of SPAI (WAP-2). Our identification of the expression of the SPAI (WAP-2) gene in a limited population of the crypt cells may also provide a useful tool for elucidating the complex and dynamic process of the epithelial differentiation.

Western blotting of porcine intestinal epithelium extracts and the authentic recombinant sample demonstrated that the molecular species of SPAI present in the intestine is the same as the authentic SPAI (WAP-2), which migrated as an about 26-kDa band on SDS-PAGE (Fig. 4). This experimentally determined value is considerably higher than the calculated value of 18 kDa for SPAI (WAP-2); the difference between the experimental and theoretical values may be due to the highly repetitive sequence in the transglutaminase substrate domain. The Western blotting result also indicates that the shorter forms of SPAI previously isolated from porcine duodenum extracts and shown to have only the WAP motif region (22) are proteolytic products artifactually generated during acid extraction and purification. Having established 1) the site of synthesis and 2) that the native *in vivo* form of SPAI has a transglutaminase substrate domain, determination of the anchoring sites and target molecules of SPAI (WAP-2) will be the subject of the next investigation.

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