Cryptic Origin of SPAI, a Plasma Protein with a Transglutaminase Substrate Domain and the WAP Motif, Revealed by \textit{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 studies on its purification and immunohistochemical localization 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 agglutinins, adhesion molecules, scorpion toxins, bacterial 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 glycylic acid 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 elucidated since its IC\(_{50}\) for renal Na\(^{+}\),K\(^{-}\)-ATPase is relatively high, around 10\(^{-6}\) \(\mu\)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 elastin, 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 elastin, 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 elastin are schematically shown in Fig. 1. In the case of elastin, its transglutaminase-mediated in vivo cross-linking to extra-cellular 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 elastin 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, \textit{in situ} hybridization and immunohistochemistry and showed that the cryoprecipitate 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 ligase kit (version 2) and Taq polymerase were from Takara, Osaka, Japan; Vecta bond 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 cDNA synthesis: 5′-ACAGCTTATAACTTCTTCCAACAC-3′ (nucleotides 483–504, Ref. 2) and 5′-AGTCGAGGTGATTCTTCTC-3′ (nucleotides 399–420, Ref. 2). The polymerase chain reaction was performed using 1 \(\mu\)g of EcoRI-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
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 EcoRI site of pBluescript II SK+

Preparation of Digoxigenin-labeled cRNA Probes—Antisense and sense RNA probes were transcribed from the template plasmid linearized by EcoRI and HindIII 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 (Boehringer Mannheim) at 1:5000. SPAI (WAP-2), used as a reference sample, was prepared 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 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 (−28 kDa, estimated by SDS-PAGE; Ref. 2).

Surface areas, roughly corresponding to the mucosal epithe-
located in the lower crypt (Fig. 2) usually not present. The SPAI (WAP-2) mRNA-positive cells case of the porcine small intestine, however, Paneth cells are
slowly migrate along the crypt-villus axis (20, 21). In the
these cells are considered to arise from multipotent stem
doxic 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. 2A) may, therefore, be enteren-
docrine cells in their early stages of differentiation. This local-
ization appears to be quite consistent with the circulatory
nature of SPAI (WAP-2). Our identification of the expression of
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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 intesti-
nal epithelium is the crypt of Lieberkuhn and its associated
villus, which contains several types of cells including 1) column-
ar absorptive enterocytes, 2) mucous goblet cells, 3) enteroen-
docrine 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
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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 artificially generated during acid extrac-
tion and purification. Having established 1) the site of synthe-
sis and 2) that the native in vivo form of SPAI has a transglu-
taminase 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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REFERENCES

1. Bairoch, A., Bucher, P., and Hofmann, K. (1996) Nucleic Acids Res. 24,
189–196
2. Kuroki, J., Hosoya, T., Takura, M., Hirose, S., Tamechika, I., Yoshimoto, T.,
Ghoneim, M. A., Nara, K., Kato, A., Suzuki, Y., Furukawa, M., and
Tachibana, S. (1995) J. Biol. Chem. 270, 22428–22433
3. McKenzie, R. M., and Larson, B. L. (1978) J. Dairy Sci. 61, 723–728
4. Araki, K., Kuroki, J., Ito, O., Kadowaki, M., and Tachibana, S. (1989)
Biophys. Res. Commun. 164, 496–502
5. Ishizuka, N., Fukushima, Y., Urayama, O., and Akera, T. (1991) Biochim.
Biophys. Acta 1089, 259–268
6. Sahara, T., Ito, F., Hagiwara, H., Saito, Y., Kuroki, J., Tachibana, S., and
Hirose, S. (1992) Biochim. Biophys. Res. Commun. 185, 240–245
7. Nara, K., Ito, O., Ito, S., Suzuki, Y., Ghoneim, M. A., Tachibana, S., and
Hirose, S. (1994) J. Biochem. (Tokyo) 115, 441–448
8. Molhuizen, H. O., Alkemade, H. A., Zeeuwen, P. L., de Jongh, G. J., Wieringa,
B., and Schalkwijk, J. (1993) J. Biol. Chem. 268, 12028–12032
9. Steinert, P. M., and Marekov, L. N. (1996) J. Biol. Chem. 270, 17702–17711
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10. Folk, J. E. (1980) Annu. Rev. Biochem. 49, 517–531.
11. Ichinose, A., Bottenus, R. E., and Davie, E. W. (1990) J. Biol. Chem. 265, 13411–13414.
12. Greenberg, C. S., Birekbbischler, P. J., and Rice, R. H. (1991) FASEB J. 5, 3071–3077.
13. Hand, D., Perry, M. J., and Haynes, L. W. (1993) Int. J. Dev. Neurosci. 11, 709–720.
14. Aeschlimann, D., and Paulsson, M. (1994) Thromb. Haemostasis 71, 402–415.
15. Parsloe, M. L. (1985) in Nucleic Acid Hybridization: A Practical Approach (Hames, B. D., and Higgins, S. J., eds) pp. 179–202, IRL Press, Oxford.
16. Tamechika, I., Itakura, M., Saruta, Y., Furrakawa, M., Kato, A., Tachibana, S., and Hirose, S. (1996) J. Biol. Chem. 271, 7012–7018.
17. Dayal, Y. (1991) in Endocrine Pathology of the Gut and Pancreas (Dayal, Y., ed) pp. 1–31, CRC Press, Inc., Boca Raton, FL.
18. Cheng, H., and Leblond, C. P. (1974) Am. J. Anat. 141, 537–562.
19. Gordon, J. I., and Hermiston, M. L. (1994) Curr. Opin. Cell Biol. 6, 795–803.
20. Ponder, B. A. J., Schmidt, G. H., Wilkinson, M. M., Wood, M. J., Monk, M., and Reid, A. (1985) Nature 313, 689–691.
21. Loeffler, M., Birke, A., Winton, D., and Potten, C. (1993) J. Theor. Biol. 160, 471–491.
22. Araki, K., Kuwada, M., Ito, O., Kuruki, J., and Tachibana, S. (1990) Biochem. Biophys. Res. Commun. 172, 42–46.
23. Wiedow, O., Schroder, J. M., Gregory, H., Young, J. A., and Christophers, E. (1990) J. Biol. Chem. 265, 14791–14795.