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Identification of Two Rodent Genes Encoding Homologues to Seminal Vesicle Autoantigen: A Gene Family Including the Gene for Prolactin-Inducible Protein

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We cloned two new paralogous genes that encode proteins homologous to seminal vesicle autoantigen (SVA) in rodents. The open reading frame of one mouse gene encodes a polypeptide consisting of 151 amino acid residues which has 43% identity to SVA. RT-PCR analysis showed selective expression in the colon, and thus the protein was tentatively named "SVA-like protein in the colon (SLP-C)". The other mouse gene has an open reading frame encoding 144 amino acid residues with 46 and 65% identity to SVA and SLP-C, respectively. Expression of this gene was detected in the mammary, submaxillary, parotid, and lacrimal glands, and this protein was named "SLP in the mammary gland (SLP-M)". Orthologs of both genes were also found in rats. The three homologous genes coding for SVA, SLP-C, and SLP-M may have been generated by gene duplication with divergence of tissue expression in the course of evolution. They comprise a unique structurally-related gene family. Moreover, these genes share significant sequence homology with that of another secretory glycoprotein, prolactin-inducible protein.

Key Words: **exocrine organs; seminal vesicle autoantigen; prolactin-inducible protein; gene duplication.**

Exocrine organs produce various proteins found in body fluids that function in host defense, nutrition, fertilization, etc. Some secreted proteins like IgA and lactoferrin are present in various body fluids such as saliva, milk, seminal plasma and tears, whereas others like prostate-specific antigen and semenogelin are specific to seminal plasma in humans (1). The seminal vesicles are part of the sexual accessory apparatus in many mammals. The function of this organ is to synthesize a variety of proteins and low molecular weight components that are secreted, stored in the lumen of the gland, and ultimately discharged with spermatozoa at the time of ejaculation.

Yu *et al.* have isolated a unique protein, called seminal vesicle autoantigen (SVA), which is specifically present in the seminal plasma of mice (2). This 19-kDa secretory glycoprotein suppresses the motility of spermatozoa by interacting with phospholipid (3). In an attempt to isolate the equivalent gene in rats, we obtained two other cDNA clones with significant homology to mouse (m) SVA. The homology search also revealed the corresponding genes in the mouse expressed sequence tag (EST) database.

In this report, we present the results of cloning and molecular characterization of the four identified genes. Furthermore, SVA and its homologues were found to share homology with another protein, prolactin-inducible protein (PIP), isolated from human breast cancer cells of the T47D cell line (4). PIP is a 15- to 17-kDa secretory glycoprotein identical to gross cystic disease fluid protein 15 (GCDFP 15) originally found by Haagensen *et al.* (5). Here we describe members of a possible PIP/SVA gene family with sequence homology and structural similarities, and speculation is offered regarding the molecular evolution of these genes.

MATERIALS AND METHODS

RT-PCR. Total RNA was extracted from the brain, heart, lung, liver, spleen, stomach, colon, kidney, submaxillary gland, lacrimal gland, seminal vesicles, testis, uterus, ovary, parotid gland, sublingual gland, prostate, epididymis, and resting mammary gland of 10-week-old ICR mice and Sprague-Dawley rats using spin columns

Abbreviations used: RT-PCR, reverse transcription PCR; RACE, rapid amplification of cDNA ends; ORF, open reading frame; EST, expressed sequence tag; SVA, seminal vesicle autoantigen; SLP, SVA-like protein; PIP, prolactin inducible protein.

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of the RNeasy Mini Kit (Qiagen). Specimens of lactating mammary gland were obtained from female mice and rats within two weeks after delivery. We designed two degenerate oligonucleotide primers based on the mSVA sequence as follows: F1, 5′-GGCN-TCNCARAAGTTYTA-3′ and F2, 5′-CYTTWSNGGNCCNTCAA-CTT-3′ (2). The first strand cDNA was synthesized using 0.5 μg of RNA from the mouse and rat tissues in a volume of 20 μl at 42°C for 30 min using AMV reverse transcriptase XL (Takara) with an anti-sense primer, M4 adapter-oligo(dT), 5′-GTTTTCCCAGTCACGACT$_n$-3′. Subsequently, PCR was performed using F1 or F2 versus the M4 primer. Amplification proceeded in a volume of 100 μl, with *Taq* DNA polymerase, through 35 rounds of temperature cycling: 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s. After labeling by means of the BigDye terminator cycle sequencing kit, the PCR products were sequenced using an ABI PRISM 310 autoanalyzer (Applied Biosystems). In experiments examining the expression of the four isolated genes, RT-PCR was employed using sequence-specific oligonucleotide primers, and β-actin cDNA was amplified as the control.

5′ RACE. We obtained the 5′ region of cDNA by RACE. Briefly, a homopolymeric tail was added to the 3′ ends of the single stranded cDNA by treatment with terminal deoxytransferase in the presence of dCTP. PCR was performed using cDNA sequence-specific primers versus a novel deoxyinosine-containing anchor primer (Gibco/BRL). When the products were not visible upon analysis by agarose gel electrophoresis, second-nested PCR was performed using inner primers corresponding to the upstream sequence.

PCR to determine the gene structure. Genomic DNA was extracted from mouse and rat liver tissue. By comparing the cDNA sequences to the genomic sequence and the organization of mSVA (6), putative exon/intron boundaries were postulated for the genes. PCR was performed using three sets of oligonucleotide primers that were designed based on the cDNA sequences for all.

DNA sequence analysis. Database searches were mainly performed with various algorithms available at the DNA DataBank of Japan (DDBJ; http://www.ddbj.nig.ac.jp). Multiple sequence alignment was performed using CLUSTAL W version 1.8 (7). A motif search was performed using the PSORT program. A phylogenetic tree was constructed by the neighbor-joining method (8), which is well-suited to cases in which the rate of nucleotide substitution differs among homologous proteins.

RESULTS AND DISCUSSION

Cloning of cDNA Homologous to mSVA Expressed in Colon Tissue

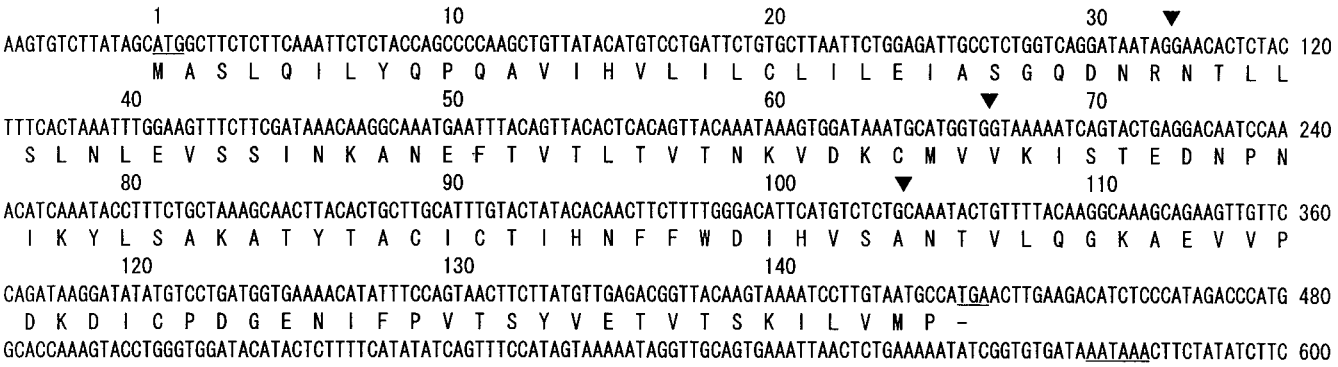
RT-PCR was performed using RNA from several rat organ tissues as the template together with two oligonucleotide primers designed based on the sequence of mSVA cDNA (2). Single products were obtained using the RNA from the colon, mammary gland and submaxillary gland as the template with the F1 and F2 primers, but no products were obtained using the RNA from the seminal vesicles. When the RT-PCR fragment derived from the rat colon was sequenced, it was found to have homology to mSVA. The sequence of the remaining region of the cDNA was determined by the 5′ RACE method. Figure 1A shows the complete nucleotide sequence of the cDNA and the deduced amino acid sequence of the putative product. The open reading frame (ORF) consists of 438 nucleotides encoding 145 amino acid residues, and the overall amino acid identity compared with the mSVA sequence was 46%. However, there was a possibility that this gene was not equivalent to the rat SVA gene assumed to exist. A homology search through BLAST algorithm revealed its partial homology to mouse EST clones from the colon, with the accession numbers AA221424, AA592866, and AA688981, which have been deposited by Marra, M., *et al.*, the WashU-HHMI Mouse EST Project at Washington University School of Medicine. Based on the reported sequence, we determined the whole cDNA sequence from the mouse clones (Fig. 1B). It consisted of 4 nucleotides comprising a 5′ untranslated region, 456 nucleotides coding for an ORF beginning with an ATG codon and terminating with a TAG codon and 133 nucleotides comprising a 3′ untranslated region. This ORF coded for 151 amino acid residues, six residues longer than that found in the rat, and these additional residues were at the carboxyl terminus. The deduced amino acid sequence of the putative protein encoded by this mouse cDNA showed 43 and 86% amino acid identity with mSVA and the putative protein encoded by the rat cDNA, respectively.

Their expression in various organs was evaluated by RT-PCR using sequence-specific primers. Expression of the homologous genes was confirmed in the colon, and no products were obtained in the case of the other organs (Fig. 2). No difference in their expression was evident comparing the findings for mouse and rat tissues. In contrast, mSVA expression was specifically limited to the seminal vesicles. Thus, the products of the two genes found to be expressed in the colon are distinct from SVA, and they are orthologous between mice and rats. The products of these newly identified genes were tentatively named “SVA-like protein in the colon (SLP-C)”. When the deduced polypeptide sequences were analyzed with the PSORT program, a putative signal peptide 28 amino acid residues in length, from Met to Gly, was recognized, and they were classified as peripheral proteins with no membrane-spanning regions. The mature proteins, mSLP-C and rat (r) SLP-C, have a calculated molecular weight of 13.6 and 13.0 kDa and an isoelectric point (pI) of 5.14 and 5.05, respectively. No potential N-linked glycosylation site (N-X-T/S) is present in the sequence of either of these polypeptides.

Cloning of cDNA for Another Homologue Expressed in the Mammary Gland

The other RT-PCR fragments amplified from the RNA obtained from rat mammary and submaxillary glands showed the same sequence and it too was homologous to the mSVA sequence, however, it was distinct from the rSLP-C sequence. Figure 1C shows the complete nucleotide sequence of the rat cDNA and the deduced amino acid sequence of the putative product. The ORF consists of 435 nucleotides encoding 144

A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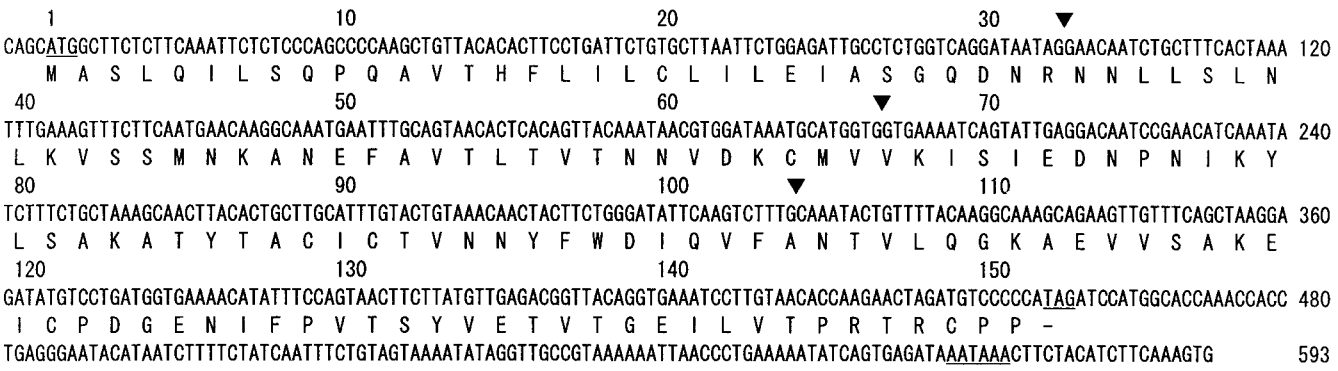
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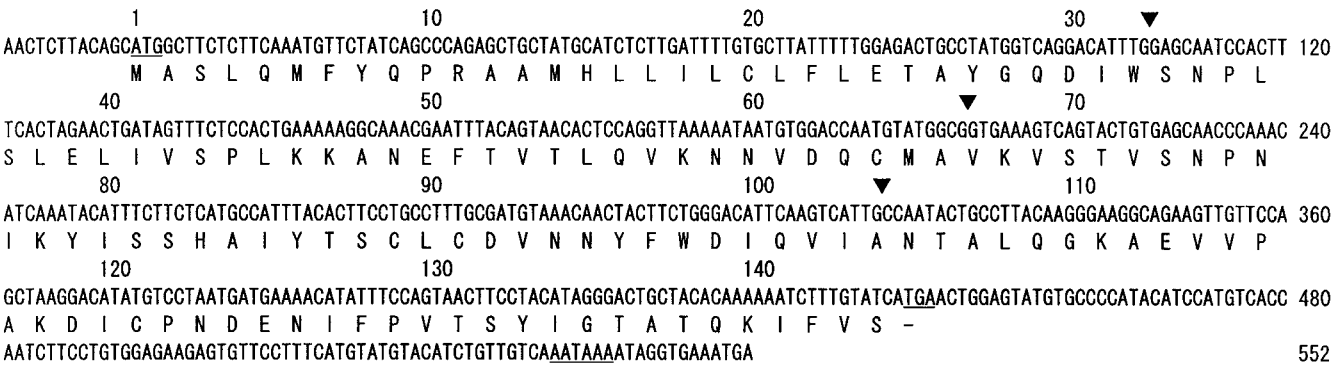
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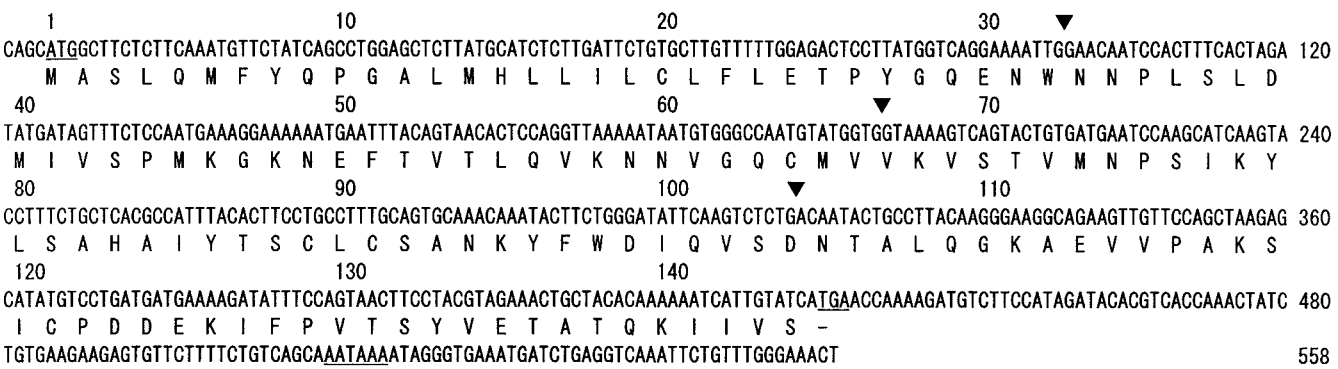
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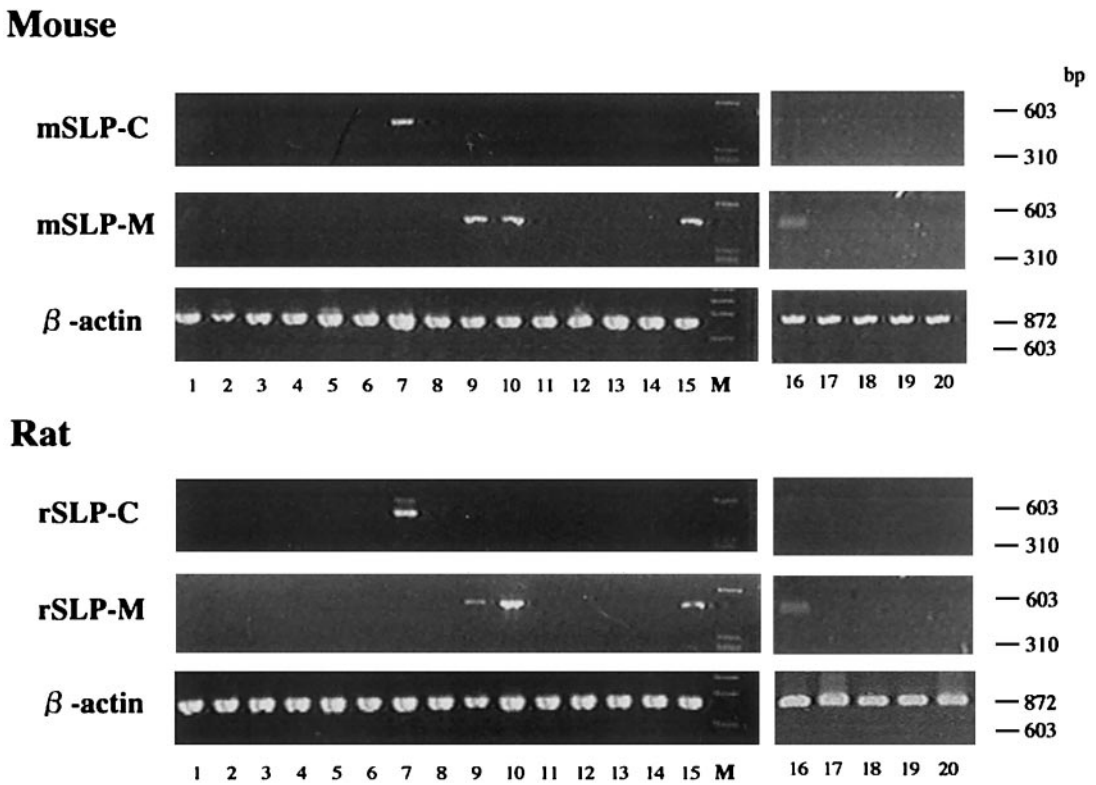

FIG. 2. RT-PCR analysis of tissue expression of the genes encoding four SVA homologues, SLP-C, and SLP-M in the mouse and rat. Lane 1, brain; 2, heart; 3, lung; 4, liver; 5, spleen; 6, stomach; 7, colon; 8, kidney; 9, submaxillary gland; 10, lacrimal gland; 11, seminal vesicle; 12, testis; 13, uterus; 14, ovary; 15, lactating mammary gland; 16, parotid gland; 17, sublingual gland; 18, prostate; 19, epididymis; 20, resting mammary gland; and M, *Hae*III-digested λDNA as molecular markers. As an internal control, β-actin cDNA was amplified.

amino acid residues, and the deduced amino acid sequence shows 40% identity with mSVA. A homology search through BLAST algorithm revealed another putative clone with homology, having the accession number BE627963, available from the mouse EST database in the Cancer Genome Anatomy Project at the National Cancer Institute. This homologous gene was also isolated from the mammary gland. The full-length cDNA sequence was obtained by the 5′-RACE and RT-PCR methods using RNA from mouse mammary gland as the template (Fig. 1D). This cDNA contained an ORF of 435 nucleotides coding for 144 amino acid residues and the deduced amino acid sequence showed 47, 65, and 80% identity with mSVA, mSLP-C and the putative product of the rat mammary cDNA isolated, respectively. When expression of these genes in various organs was studied by RT-PCR, these genes were found to be selectively expressed in the lactating mammary gland, but not in the resting one, and in the submaxillary, parotid and lacrimal glands to the greatest degree (Fig. 2). Considering the high homology and the conserved tissue expression, the proteins expressed in mammary tissue in mice and rats were thought to be orthologous, and they were named "SLP in the mammary gland (SLP-M)". A putative signal peptide 28 amino acid residues in length was evident, and the mature mSLP-M and rSLP-M proteins have a calculated molecular weight of 12.8 kDa in each instance and a pI of 6.78 and 4.95, respectively. A potential N-linked glycosylation site is present at amino acid position 76 of mSLP-M. A gene equivalent to mSVA was not found in rats in this series of experiments.

Multiple Alignment

We compared the deduced primary amino acid sequences of SLP-C and SLP-M with that of mSVA (Fig. 3). Four Cys residues that may form two tentative

FIG. 1. Nucleotide sequences of cDNA clones encoding SVA homologues and deduced amino acid sequences of the putative products. (A) cDNA derived from RNA of the rat colon (rSLP-C); (B) cDNA derived from the mouse colon (mSLP-C); (C) cDNA derived from the rat mammary gland (rSLP-M); and (D) cDNA derived from the mouse mammary gland (mSLP-M). The nucleotide and predicted amino acid residues are numbered on the right and at the top, respectively. The codons limiting the open reading frame and the putative polyadenylation signal are underlined. Filled inverse triangles (▼) indicate the positions of introns in the genes.

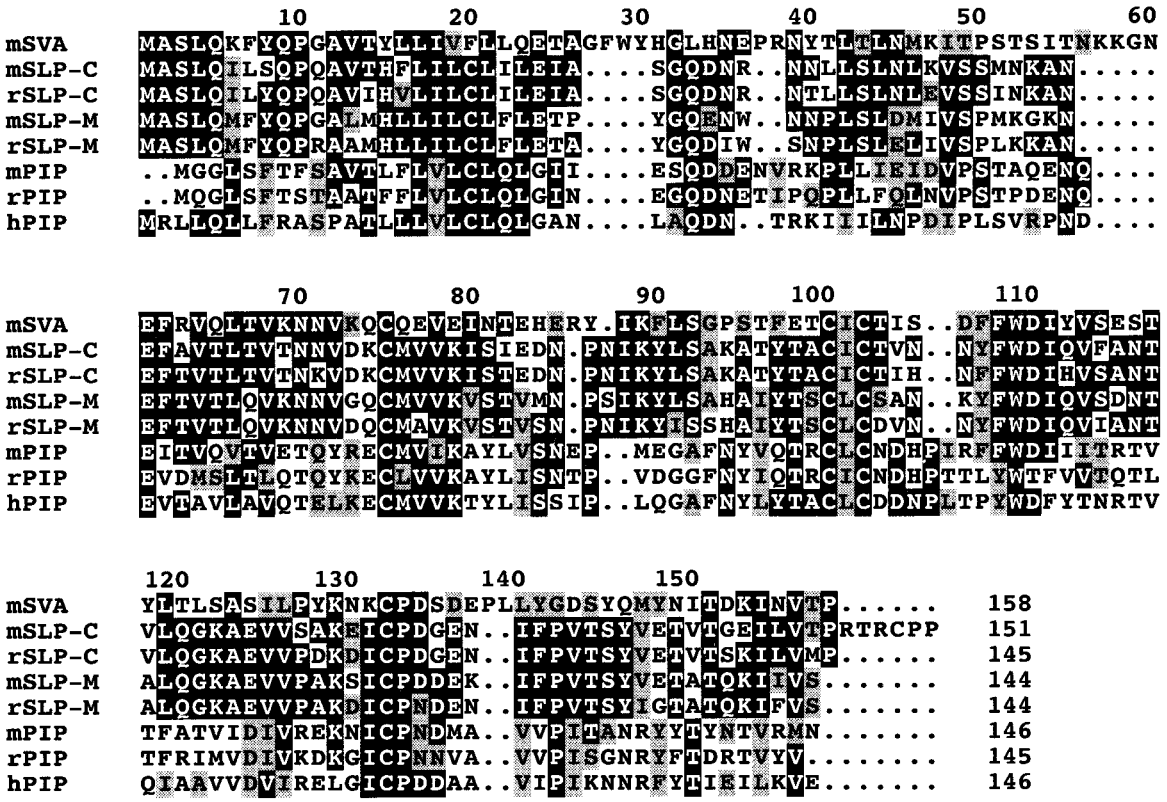

FIG. 3. Sequence alignment of the primary structures of the members of the PIP/SVA gene family. A minimum number of gaps, indicated by a dot (·), has been introduced to maximize the alignment. Identical residues at a given position are black boxed. Homologous residues (as judged from the following groups: F, Y, W; I, L, V, M; D, E; N, Q; G, A; S, T; and R, H, K) are shaded. The amino acid residues are numbered according to the ATG of mSVA. Abbreviations: m, mouse; r, rat; and h, human.

intrachain disulfide bonds in the mature proteins are completely conserved in these proteins. The mSLP-C molecule appears to have one more free Cys residue in the region of additional residues at the carboxyl terminus. Sequence identity was distributed over the lengths of the molecules. A motif sequence, 'F(Y)F-WDI', residues 107–111, is conserved in particular. Since Haung *et al.* reported that Zn^{2+} ions interact with this region of mSVA (9), the two homologues may display such zinc binding ability.

The homology search also showed that PIP, the gene for which has been cloned in the case of humans, mice and rats (4, 10, 11), is another protein with similarity to SVA, SLP-C, and SLP-M, but the degree of homology is lower than that among SVA and the two SLPs. The four Cys residues and the sequences 'CMVVK' (residues 75 to 79) and 'FFWDI' are conserved among all four of these proteins, indicating that they share structural similarity. We constructed an evolutionary tree, and SLP-C and SLP-M were placed with the two related members of this potential PIP/SVA gene family (Fig. 4). These four proteins are likely to have a common evolutionary origin, and they could be divided into two subgroups, with PIP in one subgroup and SVA, SLP-C and SLP-M in the other. It is unknown why no signal for SVA was detected in RT-PCR analysis of RNA from the rat seminal vesicles, even upon extensive analysis. Since the mSVA gene has evolved faster than the others, as shown in the phylogenetic tree, the extrapolated equivalent gene in rats might be divergent from the mouse gene.

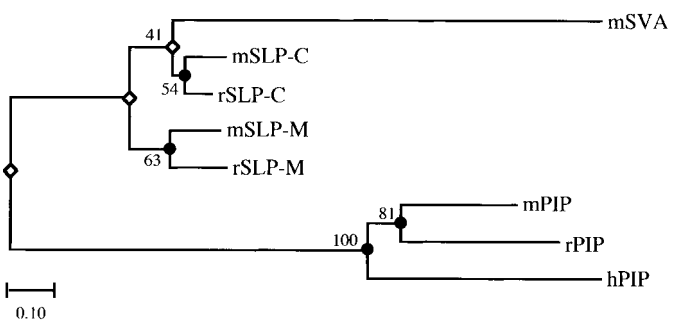

FIG. 4. Phylogenetic tree of members of the PIP/SVA gene family, constructed by the neighbor-joining method. The diamond (◇) and filled circle (●) indicate gene duplication and speciation, respectively. Although this tree became an unrooted cladogram because of no outgroups, the assumed root of this gene family was placed at the middle of the longest distance between mSVA and hPIP. The scale bar corresponds to 0.10 substitutions per site. Numbers beside the branches are bootstrap percentages on the basis of 1000 replicates.

TABLE 1

Conserved Size (bp) of Exons and Introns among SVA, SLP-C, SLP-M, and PIP

	Exon 1	2	3	4	Intron 1	2	3
mSVA	123 (95)	136	112	227 (134)	1555	1931	316
mSLP-C	>99 (95)	103	112	273 (146)	1462	2098	292
rSLP-C	>109 (95)	103	112	276 (128)	1056	1595	289
mSLP-M	>99 (95)	103	112	246 (125)	1438	1815	303
rSLP-M	>107 (95)	103	112	230 (125)	1394	1807	331
mPIP	>116 (89)	112	115	226 (125)	2232	1512	289
hPIP	137 (95)	106	115	224 (125)	~3200	~3000	290

Note. The number in parentheses indicates the number of nucleotides comprising the ORF. The genomic sequences of mSVA and hPIP referred to are those with Accession Nos. L44117 and X51501-4, respectively. The other sequence data have been deposited in the DDBJ/EMBL/GenBank database with Accession Nos. AB050099 (mSLP-C), AB050100 (rSLP-C), AB052617 (mSLP-M), AB052618 (rSLP-M), and AB017918 (mPIP).

Gene Structure

To investigate the conserved nature of the protein genes further, we determined the gene structure including the exon-intron organization in the case of each of the genes. The SVA, SLP-C, and SLP-M genes, and the PIP gene as well, commonly consisted of four exons and three intron interruptions with the GT-AG boundary consensus sequence. Table 1 summarizes the sizes of the exons and introns of the four members of this gene family (6, 12). The genes span approximately 3.6 to 7.0 kb, showing highly conserved sizes of exons and intron 3. Furthermore, independent regulatory elements such as a TATA box were identified in the 5′ flanking region in all cases, indicating that the ORFs represent the complete coding sequence and it is unlikely that alternative splicing occurs. These findings conclusively suggest that gene duplication with expressional divergence has occurred in the course of rodent evolution.

Conclusion

We isolated paralogous genes encoding homologues of mSVA which were found to be expressed in the colon and the mammary gland. Evidence of a high degree of conservation of the sequence, the disulfide bridge pattern and the gene structure indicates that the three homologous genes coding for SVA, SLP-C, and SLP-M are closely related in mice, and they may have been generated by gene duplication in the course of rodent evolution. Furthermore, they comprise a newly recognized gene family along with another glycoprotein, PIP. Since phylogenetic analysis indicated that gene duplication producing the members of the major subgroups had occurred before the evolutionary separation of humans and rodents, some counterparts of the SVA subgroup should remain in the human genome. However, it is possible that they have been lost as pseudogenes in humans.

With regard to their physiological roles, the functions of SLP-C and SLP-M remain to be elucidated. SVA is known to play a role in suppression of spermatozoa motility (3), however, the SLPs may have other functions in colon secretions, milk, saliva and tears. In contrast, the other homologous protein, PIP, has several known functions. In saliva, this protein plays a role in host defense by binding to microorganisms such as *Streptococcus* (13). PIP is an aspartyl proteinase and it acts as a factor capable of suppressing T-cell apoptosis through its interaction with CD4 (14, 15). Further studies will be necessary to fully elucidate the functions of the homologues.

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