In silico study of whey-acidic-protein domain containing oral protease inhibitors

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Abstract. Since whey-acidic-protein domain (WAP) containing protease inhibitors such as SLPI (secretory leukocyte protease inhibitor) and elafin (elastase-specific inhibitor) have antimicrobial activities and are thought to play critical roles in mucosal defenses, we are interested in these protease inhibitors. By accessing the Novartis mouse expression database, we found that the four WAP family members, SLPI, WFDC2, WFDC5, and WFDC12, are highly expressed in the oral organs, such as the trachea, tongue, and salivary glands. Since their WAP domains play pivotal roles in the antimicrobial and/or antiprotease activities and their application in therapeutics are expected to have practical value, we collected 98 WAP homologues and tried to predict their physiological functions by analyzing their amino acid sequence structures. From the multiple alignments of amino acid sequences, we predicted that most of the mammalian N-terminal WAP domains derived from SLPIs and the WAP domains derived from WFDC12s have antimicrobial activities, whereas most of the mammalian C-terminal WAP domains derived from SLPIs and the WAP domains derived from elafins have antiprotease activities. From the phylogenetic tree, it was revealed that an ancestral WAP protein initially diverged into the WFDC5-C WAP domain and the ancestral protein for the other WAP domains. Subsequently, the ancestral protein

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Abbreviations: elafin, elastase-specific inhibitor; SLPI, secretory leukocyte protease inhibitor; WAP, whey-acidic-protein; WFDC, WAP four-disulphide core; -C or -N, C-terminal WAP or N-terminal WAP

Key words: antimicrobial activity, antiprotease activity, elafin, SLPI, whey-acidic-protein, C-terminal WAP, N-terminal WAP, WFDC

for the other WAP domains diverged into two ancestral proteins, one for elafin and SLPI-C WAP domains and the other, for SLPI-N, WFDC15b, WFDC12, and WFDC5-N WAP domains, respectively. Moreover, the tree indicated that the WFDC5-N and WFDC12 WAP domains share a common ancestral protein.

Introduction

Protease inhibitors such as SLPI (secretory leukocyte protease inhibitor) and elafin (elastase-specific inhibitor) are synthesized and secreted by local tissues and/or organs and are known to have antimicrobial activities (1). These two protease inhibitors contain the whey-acidic-protein domain (WAP) and these domains are known to play pivotal roles in the antimicrobial and/or antiprotease activities (1). Since SLPI is thought to limit the spread of fungi, bacteria, and viruses on mucocutaneous surfaces *in vivo* by blocking colonization of transient pathogens and/or their release of destructive proteases such as elastase in oral sites (2), we are interested in the protease inhibitors within the oral cavity with an aim of future therapeutic applications.

We hypothesized that genes highly expressed in the tongue, salivary glands, snout epidermis, and trachea contribute to maintenance of the levels of salivary protease inhibitors. Since the expression levels of 36,182 mRNAs have been estimated for 61 different mouse organs, tissues, and/or cells by two independent experiments, and the results are stocked as a dataset in the Mouse GNF1M, we initiated our study by accessing the Novartis expression database (3), and found that four WAP family members, SLPI, WFDC2, WFDC5, and WFDC12, are highly expressed in oral organs. Subsequently, we collected 98 WAP homologues and tried to predict their physiological functions by analyzing their amino acid sequence structures. We aligned their amino acid sequences, and tried to elucidate the relationship between the amino acid sequences and their functions. We also constructed a phylogenetic tree and revealed an initial divergence of WFDC5-C-terminal WAP from an ancestral WAP protein. Based on these analyses, we discuss the relationships between the amino acid sequences and antiprotease and/or antimicrobial activities.

Oral organ (abbreviation)	Organ- specific clones	Not functionally annotated clones	Protease inhibitor clones	WAP family members		
Salivary gland (Sg)	774	214 (27.6%)	4 (0.5%)	WFDC12		
Snout epidermis (Se)	277	28 (10.1%)	5 (1.8%)			
Trachea (Tr)	186	24 (12.9%)	3 (1.6%)	WFDC2		
Tongue (To)	183	24 (13.1%)	5 (2.7%)			
To and Se	131	6 (4.6%)	4 (3.1%)			
To and Tr	25	0	5 (20.0%)	SLPI and WFDC5		
Sg and Se	5	0	0			
Sg and Tr	1	0	0			
Tr and Se	1	0	0			
Tr, Se and To	20	0	0			
Totals	1603	296 (18.5%)	26 (1.6%)	4 (0.2%)		

Table I. Classification of clones highly expressed in the oral organs.

Not functionally annotated clones include unclassifiable clones, clones coding for hypothetical proteins, and clones corresponding to DNA segments. Protease inhibitor clones include 14 serpin family members, 4 WAP family members, 2 cystatins, and 6 different protease inhibitors.

Materials and methods

Selection of oral organ-specific cDNA clones. Oral organspecific cDNA clones were selected from the expression database 'Mouse GNF1M', which contains gene expression data for 36,182 mouse genes obtained from two independent experiments using 61 organs, tissues, and/or cells (http:// wombat.gnf.org/index.html) (3). Log-transformed ratio data for the 61 organs, tissues, and/or cells were normalized. The clone was denoted as 'oral organ-specific' if the normalized ratio value exceeded +2 SD for the microarray data of the tongue, salivary gland, snout epidermis, and trachea in both of the two independent experiments (3,5).

Selection of cDNA clones coding for WAP domain containing protease inhibitors. After annotating the oral organ-specific cDNA clones, we looked for clones coding for protease inhibitors using the names of mouse protease inhibitor genes described as key words in the review of Puente and Lopez-Otin (6), and selected clones coding for WAP domaincontaining protease inhibitors using WFDC, WAP, elafin, and/or SLPI as key words.

Nomenclature. The nomenclature of primate WFDC-encoding proteins and genes are written in uppercase letters as described in Clauss *et al* (4). The corresponding proteins and genes from other vertebrates or mammals are also written with uppercase letters and numbers in this study.

Collecting the homologues of the N- and C-terminal WAP domains of mouse SLPI. We performed PSI-BLAST homology searches using either the mouse SLPI N-WAP domain with 45 amino acid residues or its C-WAP domain with 47 amino acid residues as queries against the database at the NCBI (7). After running PSI-BLAST for three iterations, we collected those clones showing an Expert (E)-value <0.001 as N-terminal

WAP homologues and those showing an E-value <0.004 as C-terminal WAP homologues. The lower the E-value, or the closer it is to zero, the more 'significant' the match is (7). The WAP domains were collected as follows: the NCBI conserved domain database (CDD version 2.1.1) (8) was downloaded and conserved domains in each WAP homologue were identified using the reverse position-specific BLAST (rpsBLAST) program (v1.65) (7). Identified domains were then extracted using the perl script named extractCD. The web version of the extractCD is available from: http://www.gen-info.osaka-u.ac.jp/~uhmin/study/extractCD/ index.html

Multiple alignments and prediction of functions carried by WAP homologues. Multiple alignments were performed with CLUSTAL W (9). Functions carried by each of the WAP homologues were predicted by comparing amino acid sequences of the WAP homologues (test sequences) to WAP domains with experimentally demonstrated antimicrobial or antiprotease activities (control sequences). After these sequences were aligned with CLUSTAL W, the levels of homology were scored by analyzing the aligned control and test sequences using the newly developed CCResid program. The scores were used to arrange the aligned sequences. The web version of the CCResid program is available from: http://www.gen-info.osaka-u.ac.jp/~uhmin/study/ccresid/ index.html

Phylogenetic tree. Phylogenetic and molecular evolutionary analyses were conducted by the neighbor-joining (NJ) method (10) in MEGA version 4.0 (11) or CLUSTAL W (9).

Results and Discussion

Isolation of cDNA clones highly expressed in the oral organs and selection of clones encoding WAP domain-containing

			Clone ID (gi numbers)				
Scientific name	Abbreviation	SLPI	WFDC5	WFDC12	Elafin		
Aotus nancymaae	NMonkey	134093146	134093142	134093143	134093144		
Bos taurus	Cattle		119906045	3132274			
Callithrix jacchus	Marmoset	134093120	134093115	134093116	134093117		
Canis familiaris	Dog		73992498		57104238		
Chlorocebus aethiops	VMonkey	134093163	134093158	134093159	134093160		
Colobus guereza	CMonkey	134093077		134093073	134093074		
Gorilla gorilla gorilla	Gorilla	134093094	134093089	134093090	134093091		
Homo sapiens	Human	4507065	21717822	119596281	4505787		
Lemur catta	Lemur	134093102	134093098	134093099	134093100		
Macaca mulatta	RMonkey	109091820	109091843	134093108	109091835		
Monodelphis domestica	Opossum		126303379				
Mus musculus	Mouse	6755574	18043977	20149778			
Ornithorhynchus anatinus	Duckbill		149637518				
Otolemur garnettii	Galago	134093085	134093082	134093083			
Ovis aries	Sheep	78126182	37528750				
Pan troglodytes	Chimpanzee	134093069					
Papio anubis	OBaboon	134093053	134093048	134093049	134093050		
Phacochoerus aethiopicus	Warthog				4887638		
Pongo pygmaeus	Orangutan	134093132	134093128	134093129	134093130		
Rattus norvegicus	Rat	5802680	27704652	57114316			
Saimiri boliviensis boliviensis	BSMonkey	134093155	134093150	134093151	134093152		
Sus scrofa	Pig				2501659		

Table II. The WAP family proteins studied.

N- and C-terminal WAP domains were extracted from each SLPI and WFDC5 sequence, whereas only one WAP domain was extracted from each WFDC12 and elafin sequence (see Materials and methods). There was one exception, the N-terminal WAP domain was not extracted from Colobus guereza SLPI (gil134093077). The WAP domain extracted from mouse WFDC15b (gil38174311) was used as one of the antimicrobial activity positive controls (see Fig. 1A), and the WAP domains extracted from chicken (gil118100817) and nematode (gil72000192) were used as out group proteins for the phylogenetic tree construction (Fig. 3).

protease inhibitors. We selected cDNA clones highly expressed in the salivary gland, tongue, trachea, and snout epidermis, and isolated 1,603 clones from 36,182 clones. We found that 296 (18.5%) of the 1,603 clones had not been functionally annotated (Table I), and determined that 26 (1.6%) of the clones encode protease inhibitors as described by Puente and Lopez-Otin (6): four of the 26 coded for WAP domain containing proteins, i.e., SLPI, WFDC2, WFDC5, and WFDC12 (4) (Table I). Since we are interested in WAP domain-containing salivary protease inhibitors with antimicrobial and/or antiprotease activities, we focused our study on these four WAP motif-containing proteins. All four of these genes are mapped on mouse chromosome 2 and share significant homology (4).

Structures and functions of WAP domains derived from SLPIs and WFDCs. To obtain insights into the relation between antimicrobial and/or antiprotease activities and the amino acid residues of the WAP domains, we collected 98 WAP homologues (Table II): 70 were derived from primates (12), 11 from rodents, 8 from ungulata, 3 from dog, 2 each from opossum and duckbill, and one each from chicken and nematode. These isolates included WAP homologues derived from SLPIs, WFDC5s, WFDC12s, and elafins (Table II). However, no WAP homologues derived from WFDC2 were included, indicating that the homologies between WAPs derived from WFDC2s and those derived from SLPIs are significantly lower. Although we were not able to isolate a cDNA clone corresponding to the mouse elafin gene from screening the Novartis mouse database, we isolated many WAP homologues derived from elafins of primates, carnivora, and ungulata in our homology search (Table II). This result is consistent with the study that most surprisingly, there is no mouse elafin gene (4).

Antimicrobial activity (Fig. 1A and B). To elucidate the relationship between the amino acid residues and protein functions, we aligned the amino acid sequences of the human SLPI-N-, mouse WFDC12-, and WFDC15b-WAP domains, because these three WAP domains have been demonstrated to have antimicrobial activities (1,13) (Fig. 1A). This alignment revealed that eight Cys residues as well as Pro11, Pro24,

(A) Positive sequences								
	10	20	30	40	50			
human SLPI	· · · · · ·	1	1		1			
Human-SLPI-N	AGVCPPKKS	AOCLRYKK	PECOSDWO	CPGKKRCCPD	TCGIKCLDPVD	Score	=	46
Mouse-WEDC12	KRVCPPDY-	VRCIRODD	POCYSDND	GDOFICCEW	OCGEKCVI PVK	Score	=	45
Mouse-WEDC15b	PCYCPEYR.	VPCPEVI I	PKCRRDKC	CKDALKCCEE	YCOMRCVDPWF	Score	-	45
mouse-millersb	**	*	* * * *	* **	* * . *	50010		45
(B) Test sequences			• • •	1 10 100				
Corilla-SIPL-N	ACVCPPKKS	AOCI RYKK	PECOSDWO	PCKKRCCPL	TCCIKCI DPVD	Score	_	46
Chimpanzoo_SIPI_N	ACVCPPKKS	AOCI PYKK	PECOSDWQ	CPCKKPCCPL	TCCIKCI DPVD	Score	2	46
Orangutan SLPL-N	ACVCPPKKS	SAOCI PVEK	PECOSDWQ	CKKRCCPL	TCCIKCI DPVD	Score	Ξ.	11
Workey SI DI N	ACVCDDVVC	AOCI DVEK	PECOSDWQ	CLCKKDCCDD	ICCINCL DRVD	Score	_	12
ORabaan SLPI N	AGVCFFRRS	AQUERTER.	PECQSDWQ	CLGKKRCCFL	ICCIKCI DRVD	Score		43
Diaboon-SLFI-N	AGVCPPKKS	AQULKIEK	PECUSDWQ	CLGKKRCCPL	ICCIKCI DRVD	Score	-	43
RMORREY-SLFI-N	AGVEPPKKS	AQULKIEK	PECOSDWQ	CLGARREEFL	ICCIKCL DDVD	Score	-	43
NMORKEY-SLPI-N	AGVCPPRKS	AULLRIEK	PECUSDWU	PEKKRUCPL	DICGIKCLDPVD	Score	=	42
BSMORKEY-SLPI-N	AGVCPPRKS	AULLRFEK	AECUSDWU	PEKKRUUPL	OVCGIKCLDPVD	Score	=	40
Marmoset-SLPI-N	AGVCPSTKP	ALULRYEK	PECUSDWU	PEKKRUCP	TUGIKULDPVD	Score	=	38
Sheep-SLPI-N	AGACPPRKS	AQCEGNEK	- PRCSSDWQ	CPHKKKCCLL	DICGIECLDPVN	Score	=	33*
Galago-SLPI-N	AGACPPRRL	AKCPKFEK	-PECRSDWQ	CPEKKRCCTL	DACSVKCLDPID	Score	=	33
Lemur-SLPI-N	AGVCPPKVP	PAPCLKKEE	RECISDWT	CPGSKRCCPH	IFCGMRCLNPH-	Score	=	31*
Mouse-SLPI-N	IGACPAKKP	PAQCLKLEK	- PQCRTDWE	CPGKQRCCQL	DACGSKCVNPVP	Score	=	31*
Rat-SLPI-N	IGACPARKE	PAQCLKLEK	- PECCTDWE	CPGKQRCCQL	DTCGFKCLNPVP	Score	=	31
						2		
Rat-WFDC12	KGVCPPDN-	VRCIRGED	- PQCHNDND	CKDQKICCYW	HCGFKCVQPV-	Score	=	36
BSMonkey-WFDC12	PGVCPADN-	VRCIKSDP	- PQCHTDQD	CQGIRKCCYL	HCGFKCVIPV-	Score	=	29
OBaboon-WFDC12	AGVCPADN-	VRCFKSDP	- PQCHTDQD	CLGARKCCYL	HCGFKCVIPV-	Score	=	29
NMonkey-WFDC12	PEVCPADN-	VRCIKSDP	- PQCHTDQD	CQGIRKCCYL	HCGFKCVIPV-	Score	=	28
Marmoset-WFDC12	PGVCPADN-	IRCIKSDP	- PQCHTDQD	CQGIRKCCYL	HCGFKCVIPV-	Score	=	28
Orangutan-WFDC12	AGVCPADN-	VRCFKSDP	- PQCHTDQD	CLGERKCCYL	HCGFKCVIPV-	Score	=	28
VMonkey-WFDC12	AGVCPADN-	VRCFKSDP	- PQCHTDQD	CLGERKCCYL	HCGFKCVIPV-	Score	=	28
Gorilla-WFDC12	AGVCPADN-	VRCFKSDP	- PQCHTDQD	CLGERKCCYL	HCGFKCVIPV-	Score	=	28
Human-WFDC12	AGVCPADN -	VRCFKSDP	- PQCHTDQD	CLGERKCCYL	HCGFKCVIPV-	Score	=	28
CMonkey-WFDC12	AGVCPADN-	VRCFKSNP	- PQCHTDQD	CLGERKCCYL	HCGFKCVIPV-	Score	=	27
RMonkey-WFDC12	AGVCPADN-	IRCFKSDP	- POCHTDOD	CLGERKCCYI	HCGFKCVIPV-	Score	=	27
Lemur-WFDC12	WGNCPAEK-	GSCIKSGP	-SQCHADND	CPGDKKCCFL	SCSFKCVSPD-	Score	=	25
Duckbill-WFDC5-N	LGGCPADD-	TPCLWORP	-DOCSEDSO	CPRLKKCCSF	ACYROCLPPVR	Score	=	22*
Opossum-WFDC5-N	SGGCPPDD-	OVCRPGIP	-HOCLVDKO	CPKGKKCCO	ACFLOCVA	Score	=	21*
NMonkey-WFDC5-N	PGACPPDD-	GPCLLSVP	-DOCMEDRO	CPLTRKCCYF	RACFROCVPR	Score	=	20*
Galago-WFDC5-N	PGGCPPDD-	GPCLLSVP	- DOCTDDSO	CPSTMKCCP	RACFROCIPR	Score	=	20*
Lemur-WFDC5-N	AGACPPED-	GPCVISVP	-DOCOEDSO	CPSTMKCCFC	ACFRHCVPR	Score	=	20*
Human-WFDC5-N	SGCCPPDD-	GPCLLSVP	- DOCVEDSO	CPLTRKCCY	RACFROCVPR	Score	=	19*
Gorilla-WFDC5-N	SGGCPPDD-	GPCLLSVP	-DOCVEDSO	CPLTRKCCY	ACFROCVPR	Score	=	19*
BSMonkey-WFDC5-N	SGGCPPDD-	GPCLLSVP	-DOCMEDSO	CPWTRKCCYK	ACFROCVPR	Score	=	19*
Marmoset-WFDC5-N	SGRCPPDD-	GPCLLSVP	-DOCMEDSO	CPETRKCCY	ACFROCVPR	Score	_	19*
Rat-WFDC5-N	LGGCPPDD-	GPCSOVIP	-DOCANDKO	CPSSWKCCS	ACELOCMPR	Score	_	19*
Cattle-WFDC5-N	SGCCPPDD-	GPCLISVP	-DOCLHDSO	CPSGMKCCR	CCFLOCVR	Score	_	19*
Dog-WFDC5-N	EGGCPPDD.	GPCLISVP	-DHCMDDSO	CPARMKCCSK	ACEROCVPM	Score	-	18*
Mouse-WEDC5-N	LGGCPPDD.	GLCNONTP	PDOCI NDKO	CPSSWKCCR	RACEL OCMPR	Score	-	18*
VMonkey-WEDC5-N	WCCCPADD	GPCLISVP	-DOCVEDSO	CPLTRKCCVI	ACEROCVPR	Score	-	18*
OBaboon-WEDC5-N	WCCCPADD.	CPCLI SVP	DOCVEDSO	CPLTRKCCV	ACEROCVPR	Score	-	18*
RMonkey-WEDC5-N	WCCCPADD	GPCLI SVP	DOCVEDSO	CPLTRKCCVI	ACEROCIPR	Score	_	17*
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Figure 1. Multiple sequence alignment of WAP homologues to the N-terminal WAP domain of mouse SLPI. (A) Positive sequences show alignments of WAP domains demonstrated to have antimicrobial activities. (B) Test sequences show alignments of SLPI-N-WAPs, WFDC12-WAPs, and WFDC5-WAPs. Multiple alignments were performed as described in Materials and methods. For the conservation line output in the CLUSTAL format alignment file, three characters are used: '*' indicates positions that have a single, fully conserved residue; ':' and '.' indicate positions that contain substitutions with highly and with weakly similar amino acid residues, respectively. Red boxes indicate fully conserved residues; yellow boxes, amino acids present within the positive sequences; non-colored boxes, amino acids substituted with highly or weakly similar amino acid residues. The alignments were arranged according to the arbitrarily defined scores: each red or yellow box for an amino acid is scored as 1 and each grey or non-colored box is scored as zero. The scores marked with ** indicate that the WAPs are predicted not to have similar antimicrobial activities. The underlined amino acid residues correspond to the conserved amino acid residues substituted with unrelated amino acid residues. Names, origins, and abbreviations related to the WAP domains are summarized in Table II. Amino acid residue numbers of human SLPI are shown in panel (A).

Asp29, and Pro50 were completely conserved and that Ala16, Glu25, Gly34, Ile45, Lys46, Leu48, and Asp52 of human SLPI were all replaced by similar amino acid residues among these three WAP domains. Most of these conserved amino acid residues should have pivotal roles in the antimicrobial activities.

Subsequently, we aligned the amino acid sequences of 14 SLPI-N-, 12 WFDC12-, and 16 WFDC5-N-WAP domains (Fig. 1B). The results obtained with 14 SLPI-N- and 12 WFDC12-WAP domains revealed that they maintained nearly

all of the above-described conserved amino acid residues (Fig. 1A and B). There were three exceptions: i) Pro24 of Human-SLPI-N was replaced by Arg24 in Lemur-SLPI-N; ii) Ile45 of Human-SLPI-N was replaced by Thr45 in Sheep-SLPI-N, and iii) by Ser45 in Mouse-SLPI-N (see Fig. 1B, scores marked with **). These results suggest that the remaining 23 of 26 SLPI-N- and WFDC12-WAP domains retain antimicrobial activities.

On the other hand, although the eight Cys residues were conserved among the 16 WFDC5-N, several other above-

(A) Positive sequences								
	70	80	90	100				
human SLPI	1	1	1	1				
Human-SLPI-C	PGKCPVTYGOCL	LNPPNFCEMD	GOCKRDLKC	CMGMCGKSCVSPVK	Score	=	46	
Sheep-SLPI-C	PGTCPVIHGOCL	ILKPLNHCETD	DOCIGALKC	CKAMCGKVCLSPVK	Score	=	46	
Mouse-SLPI-C	PGRCVKTOARCM	LNPPNVCORD	GOCDGKYKC	CEGICGKVCLPPM-	Score	=	45	
Human-elafin	PGSCPIILIRCAM	LNPPNRCLKD	TDCPGIKKC	CEGSCGMACFVPO-	Score	=	45	
Pig-elafin	PGSCPRILIRCLM	IVNPPNRCLSD	AOCPGLKKC	CEGFCGKACMDPK-	Score	=	45	
	** * :* *		* **	* . ** * . *				
human elafin	1	1						
	20	30	40	50				
(B) Test sequence:	S							
Chimpanzee-SLPI-C	PGKCPVTYGQCLM	LNPPNFCEMD	GQCKRDLKC	CMGMCGKSCVSPVK	Score	=	46	
Gorilla-SLPI-C	PGKCPVAYGQCLM	ILNPPNFCEMD	GQCKRNLKC	CMGMCGKSCVSPVK	Score	=	44	
Orangutan-SLPI-C	PGKCPVAYGQCLM	ILNPPNYCEMD	GQCERDLKC	CMGMCGKSCVSPVK	Score	=	43	
RMonkey-SLPI-C	PGRCPPAYGQCMM	ILKPPNYCEMD	GQCERDLKC	CMGMCGKSCVSPVK	Score	=	42	
OBaboon-SLPI-C	PGRCPPAYGQCMM	ILKPPNYCEMD	GQCERDLKC	CMGMCGKSCVSPVK	Score	=	42	
VMonkey-SLPI-C	PGRCPPAYGQCMM	LNPPNYCEMD	GQCERDLKC	CMGMCGKSCVSPVK	Score	=	42	
CMonkey-SLPI-C	PGRCPLAYGQCMM	ILNPPNYCEMD	GQCERDLKC	CVGMCGKSCVSPVK	Score	=	41	
Rat-SLPI-C	PGRCVKFQGKCLM	ILNPPNKCQND	GQCDGKYKC	CEGMCGKVCLPPV-	Score	=	41	
BSMonkey-SLPI-C	AGKCPAVYGQCMM	INPPNHCESD	GQCERDLKC	CMGMCGKTCVSPL-	Score	=	38	
Galago-SLPI-C	PGACPVVHGRCMM	INPPNHCERD	GQCQDDFKC	CMGMCGKACVSPT-	Score	=	38	
NMonkey-SLPI-C	SGKCPVVYGQCMM	IDPPNHCELD	GQCERDLKC	CVGMCGKTCVSPL-	Score	=	36	
Marmoset-SLPI-C	PGKCPVVYGQCMM	IDPPNHCESD	GQCEHDMKC	CIGMCGKTCVSPL-	Score	=	36	
Lemur-SLPI-C	PGTCPAVYGYCMM	ILNPSNSCESD	GQCQAKFKC	CVGMCGKIC <u>S</u> SPVE	Score	=	34**	
Gorilla-elafin	PGSCPVILIRCAM	ILNPPNRCLKD	TDCPGIKKC	CEGSCGMACFVPQ-	Score	=	45	
OBaboon-elafin	PGSCPNILIRCAM	LNPPNRCLKD	TDCPGIKKC	CEGSCGMACMVPQ-	Score	=	44	
RMonkey-elafin	PGSCPNILIRCAM	LNPPNRCLKD	TDCPGIKKC	CEGSCGMACMVPQ-	Score	=	44	
CMonkey-elafin	PGSCPNILIRCAM	LNPPNRCLKD	TDCPGIKKC	CEGSCGMACMVPQ-	Score	=	44	
VMonkey-elafin	PGSCPNILIRCAM	LNPPNHCLKD	TDCPGIKKC	CEGSCGMACMVPQ-	Score	=	44	
NMONKey-elafin	SGSCPKILIRCAM	LNPPNRCLKD	TDCPGIKKC	CEGSCGMACLDPQ-	Score	=	44	
BSMONKey-elafin	SGSCPKILIQCAM	ILNPPNRCLKD	TDCPGIKKC	CEGSCGMACLDPQ-	Score	=	44	
Urangutan-elafin	PGFCPNILIRCAM	LNPPNRCLKD	TDCPGIKKCO	CEGSCGMACFVPQ-	Score	=	43	
Marmoset-elaiin	SGSCPKILIRCAM	LNPPNRCLKD	TDCPGIKKC	CEGSCGMACLDPR-	Score	=	43	
Galago-elafin	SGSUPNILIRUAM	UNPPNHULSD	TUCPGTKKC	LEGSCGKRCMDPQ-	Score	=	41	
warthog-elafin	PGSCPRILIRUMM	INNPPNKULSU	AUCPGVKKC	LEGFLGKELLNPR-	Score	=	41	
Sneep-erarin	DCSCDNIL IDCAN	MNPPNKULKD	AUCPGAKKU	EGSCGRACINDO	Score	=	41**	
Dog olafin	POSCENILIRCAM	LNPPNRC65D	TECHCARKC	CEGSUGRAULNPQ-	Score	=	39	
Cattle-Trappin-6	PCICPDVDIHCNI	WNDDNOCWDD	AHCDCAKKC	CECECCETCUNED	Score	=	39	
cattle-mappin-o	KGLCFRVRINCNL	MALLACAKD	ANCEGARACI	LEGFLGKICMNPR-	score	=	31++	
NMonkey-WEDC5-C	LOSCPEDOL ROLS	PMN_HI CHKD	ADCSCKKPC	QUADES ACCEDICEDENE	Score	-	25**	
Mouse-WEDC5-C	SCKCPVDOL RCL	PTK-HMCNKD	SDCSCKKRC	CASACCEDCEDES	Score	-	20	
OBaboon-WEDC5-C	PGSCPODOL RCL	PMN-HI CHKD	SDCSCKKRC	CHSACCRDCRDPAR	Score	-	24**	
RMonkey-WEDC5-C	PGSCPODOL RCL	PMN-HI CHKD	SDCSCKKRC	CHSACCEDCEDEAR	Score	-	24	
VMonkey-WFDC5-C	PCSCPODOL RCL	PVN-HOCHKD	SDCSCKKPC	CHSACCPDCPDPAR	Score	_	24**	
BSMonkey-WFDC5-C	LGSCPEDOL RCLS	PMN-HI CHKD	ADCSCKKRC	CPSACCRDCRDPAR	Score	_	24**	
Marmoset-WFDC5-C	LGSCPEDOLOCUS	PMN-HI CHKD	ADCSCKKRC	CPSACCRDCRDPAR	Score	-	24**	
Orangutan-WFDC5-C	LGSCPEDOL RCL S	PMN-HI CHKD	SDCSCKKRC	CHSACCRDCRDPAR	Score	_	23**	
Human-WFDC5-C	LGSCPEDOLRCLS	PMN-HLCHKD	SDCSGKKRC	CHSACGRDCRDPAR	Score	-	23**	
Rat-WFDC5-C	LGKCPVDOLHCLS	PRK-HLCDKD	LDCSGKKRC	CVSACGRDCRDPS-	Score	_	23**	
Galago-WFDC5-C	LGSCPVDOLHCLS	PTK-HLCHOD	SNCSGKKRC	CPTACGRDCRDPVK	Score	_	23**	
Lemur-WFDC5-C	RGGCPEDOLOCLS	PTE-HLCNKD	SDCSGKKRC	CHTSCGRECRDPAR	Score	_	23**	
Duckbill-WFDC5-C	LGDCPKDDLLCLS	PIO-HLCCKD	TDCSGIOKC	CLAACGRDCREPAK	Score	=	23**	
Gorilla-WFDC5-C	LGSCPEDOLHCLS	PMN-HLCHKD	SDCSGKKRC	CHSACGRDCRDPAR	Score	=	22**	
Dog-WFDC5-C	PGSCPEDRLOCLS	PVQ-HLCHKD	ADCRGSSRC	CLGACGRDCRNPI -	Score	=	22**	
Cattle-WFDC5-C	TGRCPEDRLRCVS	PVO-HLCSKD	SDCOGRKRC	CPGACGRDCRNPV-	Score	=	21**	
Opossum-WFDC5-C	SGQCPPDPLRCLS	ATQ-HLCTOD	KNCSGLKRC	CPTACGRDCRDPVR	Score	=	20**	

Figure 2. Multiple sequence alignment of WAP homologues to the C-terminal WAP domain of mouse SLPI. (A) Positive sequences show alignments of WAP domains demonstrated to have antiprotease activities. (B) Test sequences show SLPI-C-WAPs, elafin-WAPs, and WFDC5-C-WAPs. The other procedures, abbreviations, and symbols are as described in Fig. 1 and/or in Table II. Amino acid residue numbers of human SLPI and those of human elafin are shown in panel (A).

described conserved amino acid residues were replaced by unrelated residues. For example, out of the 16 WFDC5-N-WAPs, Pro24 of Human-SLPI-N was replaced by Asp24 in all 16 WAPs, Ile45 of Human-SLPI-N was replaced by Arg45 in 12 WAPs, and Pro50 of Human-SLPI-N was replaced by Arg50 or Met50 in 14 WAPs (Fig. 1A and B). These results suggest that although the 16 WFDC5-N-WAP domains have conserved tertiary structures, they do not have similar antimicrobial activities, or have antimicrobial activities against different microbes. Antiprotease activity (Fig. 2A and B). Grutter et al (14) found that human SLPI contacts proteases through Leu72 and Met73 of the 'primary' binding loop, which consists of eight residues from Thr67 to Leu74 (Fig. 2A, SLPI). Tsunemi et al (15) described the primary binding site of human elafin, the V region, to consist of Ala24, Met25, and Leu26, which correspond to Leu72, Met73, and Leu74 of human SLPI (Fig. 2A, elafin).

Since the human SLPI-C-WAP and WAP domains derived from human and pig elafins have antiprotease activities, we



Figure 3. A phylogenetic tree of WAP domains. This tree was constructed by aligning the amino acid sequences of the WAP homologues. Phylogenetic and molecular evolutionary analyses were conducted using the MEGA4 program as described in Materials and methods. Bootstrap values obtained from 1,000 replicates are shown at the corresponding branches by the 1/10 values. The scale bar represents an estimate of the number of amino acid residue substitutions per site. Green colored WAPs are predicted to have antimicrobial activities and yellow-colored WAPs are predicted to have antiprotease activities (see Figs. 1 and 2). The other abbreviations are as described in Table II.

aligned the amino acid sequences of these three WAP domains (1,16) (Fig. 2A). We added the C-WAP domains derived from mouse and sheep SLPIs to this alignment because these two SLPIs have also been demonstrated to have similar antiprotease activities (17,18). The alignment revealed that eight Cys residues as well as Pro61, Gly62, Met73, Pro76, Asn78, Asp83, Lys91, Gly98, and Pro104 were all conserved, and

that Gln70, Leu74, Asn75, Gln85, Gly94, and Val102 of human SLPI were all replaced by similar amino acid residues in the other 4 sequences. All these conserved amino acid residues should have pivotal roles in the antiprotease activity (Fig. 2A).

We collected 13 SLPI-C, 15 elafin, and 17 WFDC5-C WAP domains, and aligned their amino acid sequences

(Fig. 2B). The results obtained with the 13 SLPI-C and 15 elafin WAP domains revealed that they retained almost all of the above-described conserved amino acid residues (Fig. 2A and B). There were three exceptions: i) Gln70 and Val102 of Human-SLPI-C were replaced by Tyr70 and Ser102, respectively, in the Lemur-SLPI-C WAP domain; ii) Pro61 of human elafin was replaced by Arg61 in Sheep-elafin, and iii) Pro61 and Leu74 of Human-SLPI-C were replaced by Arg61 and Trp74, respectively, in Cattle-Trappin-6. Accordingly, we predict that Lemur-SLPI-C-, Sheep-elafin, and Cattle-Trappin-6 WAP domains do not retain antiprotease activities (see Fig. 2, scores marked with **). Although Pro61 of Human-SLPI-C was replaced by Ala61 in BSMonkey-SLPI-C, by Ser61 in Nmonkey-SLPI-C, and by Ser61 in Nmonkey-, BSMonkey-, Marmoset-, and Galagoelafins, these are similar amino acid residues, and these results suggest that these proteins keep their antiprotease activities.

On the other hand, although the eight Cys residues were completely conserved among the 17 WFDC5-C, the amino acid residues of the regions corresponding to the 'primary' binding site of human SLPI (Leu72, Met73, and Leu74 region) and/or to the V region of human elafin (Ala24, Met25, and Leu26 region) were replaced by different amino acid residues (Fig. 2B) (13,14). These results suggest that the 17 WFDC5-C-WAP domains either have antiprotease activities against different proteases, or do not have antiprotease activities.

Evolution of the WAP domains. To elucidate the evolutionary relationship among WAP domains, we constructed a phylogenetic tree using the amino acid sequences of the WAP domains shown in Figs. 1 and 2 (Fig. 3). Our analysis revealed that an ancestral WAP protein diverged into the WFDC5-C-WAP domain and the ancestral protein for the other WAP domains. Brown et al (16) showed a close relationship between elafin-WAP and SLPI-C-WAP domains, and suggested a divergence in their evolution from an ancestral protein subsequent to the divergence of SLPI-N- and SLPI-C-WAP domains. Our results not only support this concept, but also suggest a divergence of WFDC15b-, WFDC12-, and WFDC5-N-WAP domains from a diverged ancestral SLPI-N-WAP domain (Fig. 3). Moreover, our results suggest that WFDC5-Nand WFDC12-WAP domains share a common ancestral protein (Fig. 3).

Possible applications in therapeutics. Antimicrobial properties of recombinant human SLPI-N-WAP are observed not only *in vivo* against *E. coli* and *Staphylococcus aureus*, but also *in vitro* against the spread of fungi, bacteria, and viruses (1,2). Since elderly individuals are particularly susceptible to mucosal infections and are known to produce diminished amounts of oral SLPI (2), these studies suggest that supplying recombinant WAPs to the saliva of elderly patients could prevent mucosal infections. Apparently, these recombinant proteins have advantages over many other protease inhibitors because they are not glycosylated and are much smaller (~5 kDa) than natural protease inhibitors such as serpins (40-50 kDa) (19) and cystatins (11-175 kDa) (20).

In summary, we have isolated 98 WAP homologues and based on their multiple alignments, we predict that most

mammalian SLPI-N- and WFDC12-WAP domains have antimicrobial activities, whereas most mammalian SLPI-C- and elafin WAP domains have antiprotease activities. We also discussed the evolution of WAP family members. According to Fitch *et al* (1), increasingly virulent strains of bacteria and antibiotic resistance mean that proteinase inhibitors have the capacity to become the next 'magic bullet'. We are therefore continuing our efforts to collect WAP homologues, analyze their amino acid sequences, and predict *in silico* whether these homologues could have novel antimicrobial and/or antiprotease activities.

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