

Phylogenetic Analysis of the Homologous Proteins of the Terminal Complement Complex Supports the Emergence of C6 and C7 Followed by C8 and C9

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Abstract. The plasma complement system comprises several activation pathways that share a common terminal route involving the assembly of the terminal complement complex (TCC), formed by C5b-C9. The order of emergence of the homologous components of TCC (C6, C7, C8 α , C8 β , and C9) has been determined by phylogenetic analyses of their amino acid sequences. Using all the sequence data available for C6-C9 proteins, as well as for perforins, the results suggested that these TCC components originated from a single ancestral gene and that C6 and C7 were the earliest to emerge. Our evidence supports the notion that the ancestral gene had a complex modular composition. A series of gene duplications in combination with a tendency to lose modules resulted in successive complement proteins with decreasing modular complexity. C9 and perforin apparently are the result of different selective conditions to acquire pore-forming function. Thus C9 and perforin are examples of evolutionary parallelism.

Key words: Evolution — Complement system — Terminal complement complex — Membrane attack complex — Perforin

Introduction

The terminal half of the complement cascade is a common pathway shared by all the pathways of complement activation. The proteins involved, C5, C6, C7, C8 α , C8 β , and C9, with the exception of C5, are comprised of several conserved cysteine-rich modules and a cysteine-poor region. Interestingly, the γ chain of C8 does not participate in the formation of the C5b-9 complex (Brickner and Sodetz 1984). Although C5b-9 is often called the membrane attack complex (MAC), this martial term is appropriate only for the defense against Neisseria pathogens and for the pathologic effects of C5b-9. It is more accurate to refer to C5b-9 as a terminal complement complex (TCC), because it is normally involved in signaling to host cells (reviewed by Nicholson-Weller and Halperin 1993). C5b-9 can insert into membranes and interact directly with G proteins, thus effecting signaling in the absence of a specific receptor (Niculescu et al. 1994). The first step in activating C5b-9 is to activate C5. While the amino acid sequence of C5 is homologous to that of C3 and C4 (Nonaka and Takahashi 1992), C6, C7, C8 α , C8 β , and C9 are homologous to perforin, the lytic protein of NK cells and cytotoxic lymphocytes (Young et al. 1986a). The similarity in size, sequence and function between perforin and C9 (reviewed by Tschopp et al. 1986; Young et al. 1986b), led to the hypothesis that C9 emerged from the original duplication of the common ancestral gene to perforins and to TCC

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Table 1. Matrix of identity and distance values between C6-C9 and the perforins^a

	GenBank	Puffer C0	Trout C0	Det CO	Mouse C0	Pabbit CO	Horac CO	U
	reference	Puffer C9	Trout C9	Rat C9	Mouse C9	Rabbit C9	Horse C9	Human C9
Puffer C9	1845349		68.3	48.44	47.29	47.74	48.16	47.59
Trout C9	116616	0.318	_	49.58	48.44	47.47	48.45	47.61
Rat C9	1256828	0.516	0.504		80.94	77.56	80.33	78.95
Mouse C9	755764	0.527	0.516	0.191		71.87	74.37	72.14
Rabbit C9	1352109	0.523	0.525	0.224	0.281	_	81.87	81.04
Horse C9	1352107	0.518	0.515	0.197	0.256	0.181	_	87.91
Human C9	1352108	0.524	0.524	0.211	0.279	0.19	0.121	
Rabbit C8a	1352105	0.65	0.649	0.613	0.594	0.614	0.588	0.614
Human C8a	729167	0.657	0.646	0.606	0.598	0.602	0.59	0.61
Rabbit C8B	1352106	0.675	0.665	0.62	0.616	0.641	0.634	0.64
Human C8B	116612	0.658	0.659	0.605	0.607	0.617	0.628	0.628
Human C6	116609	0.644	0.65	0.639	0.624	0.632	0.641	0.635
Human C7	87197	0.656	0.645	0.612	0.622	0.609	0.615	0.612
Mouse perforin	110800	0.712	0.727	0.733	0.718	0.725	0.725	0.74
Rat perforin	548477	0.72	0.727	0.733	0.725	0.74	0.74	0.756
Human perforin	129819	0.735	0.727	0.74	0.733	0.733	0.74	0.756

^a The identity values computed by MEGA appear above the diagonal. The distance values under the diagonal were calculated by PAUP to reconstruct the phylogeny shown in Fig. 4.

proteins (Podack et al. 1988). According to this hypothesis C8, C7, and C6 should have successively emerged from C9 through later gene duplication events. Recently, a more complex evolutionary history for the TCC components has been advanced, proposing that the ancestral protein was a C6/C7-like molecule (Hobart et al. 1993, 1995; Hobart 1998) and that the evolution of these proteins proceeded from the complex to the simple. Despite the fact that more than a dozen amino acid sequences of C6–C9 are already available, no phylogenetic analysis has been reported to test the two hypotheses. Therefore, we carried out standard analyses with all available amino acid sequences of C6-C9 and performs as outgroups. Reconstructed trees suggest that C6 and C7, which share the greatest similarity to each other (Haeflinger et al. 1989; DiScipio et al. 1988), were the earliest TCC components to emerge.

Materials and Methods

Phylogenetic Analysis of Amino Acid Sequences. Phylogenetic analyses were based on the amino acid sequence similarity of C6, C7, C8 α , C8 β , C9 (C6–C9), and perforin. All of these are mosaic proteins formed by a set of one to eight cysteine-rich modules of 30–70 amino acids each and one central cysteine-poor region of approximately 350 residues (González et al. 1996). The cysteine-rich regions include modules that show similarity to sequences in other proteins, namely, thrombospondin, low-density lipoprotein receptor, epidermal growth factor, complement factor I, and the short consensus repeats present in the "regulators of complement activation" gene cluster of the complement control proteins (Reid and Day 1989). The epidermal growth factor module and a partial cysteine-poor region are also present in perforin.

The amino- and carboxyl-terminal thrombospondin, low-density lipoprotein receptor, cysteine-poor, and epidermal growth factor modules of all amino acid sequences available for C6–C9 in the GenBank

database, as well as the cysteine-poor and epidermal growth factor sequences of human, rat, and mouse perforins, were initially aligned using the Clustal algorithm (Higgins and Sharp 1988) in DNAMAN (Lynnon BioSoft 1994-1997) and then adjusted by eye. Sequences included Homo sapiens (human) C6, C7, C8a, C8B, and C9; Oryctolagus cunniculus (rabbit) C8a, C8B, and C9; and Rattus norvegicus (rat), Mus musculus (mouse), Equus caballus (horse), Fugu rubripes (puffer fish), and Oncorhynchus mykiss (rainbow trout) C9. A branchand-bound parsimony analysis was carried out by 100 bootstrap replicates with a prerelease version (4.0.0d61a) of PAUP (Swofford 1990), using the perforins as outgroups. Only the thrombospondin, lowdensity lipoprotein receptor, and epidermal growth factor modules plus the cysteine-poor region were included in the analysis because they are present in all the TCC proteins and can be reliably aligned, excluding all sites for which positional homology is uncertain (Swofford et al. 1996) (Fig. 1). All other conditions were set at "default" for PAUP analysis. Distance phylogenetic analyses were also performed with PAUP by neighbor-joining searches in a bootstrap of 100 replicates. The distance measure is equal to the mean character difference. All other conditions were set at "default."

Relative Rate Test. Evolutionary rates of change of performs and TCC proteins were evaluated using the two-cluster relative rate test (Takezaki et al. 1995) for the Poisson pairwise distances of the aligned sequences. The test was carried out on all sequences that were used for phylogenetic analysis, as well as on individual modules; gaps within sequences were always excluded. The relative rate test calculations were performed using the program PHYLTEST (Kumar 1996). In this test, L_a and L_b are the averages of observed numbers of substitutions per site from the common ancestor of each sequence cluster; then $L_a = L_b$ is the null hypothesis under the constancy of the molecular clock, i.e., $\delta = L_a - L_b = 0$. Because the variance of δ can be estimated, the deviation of δ from 0 and thus the constancy of evolutionary rates between lineage A and lineage B could be examined with a two-tailed normal deviate test, where the statistic Z should not be larger than 1.96 to accept the rate constancy (null hypothesis) at the 5% level.

Statistic Analyses. Identity was estimated by pairwise comparison of the sequences used for phylogenetic analysis as well as of individual

Rabbit C8a	Human C8α	Rabbit C8β	Human C8 _β	Human C6	Human C7	Mouse perforin	Rat perforin	Human perforin
34.9	34.2	32.43	34.06	35.54	34.32	28.03	27.27	25.76
34.97	35.32	33.42	33.97	34.92	35.41	26.52	26.52	26.52
38.66	39.36	37.96	39.51	36.14	38.77	25.95	25.95	25.19
40.64	40.18	38.39	39.32	37.58	37.77	27.48	26.72	25.95
38.55	39.83	35.89	38.34	36.83	39.14	26.72	25.19	25.95
41.16	40.99	36.62	37.23	35.93	38.53	26.72	25.19	25.19
38.55	38.95	36.	37.23	36.53	38.84	25.19	23.66	23.66
	83.7	42.74	42.47	41.18	37.06	30.3	30.3	30.3
0.161	_	42.47	42.2	40.91	38.42	29.55	31.06	28.79
0.571	0.574	_	87.66	37.77	36.24	24.44	25.19	25.19
0.574	0.577	0.121	_	38.32	36.8	25.19	25.93	25.93
0.587	0.59	0.621	0.616	_	43.8	31.85	31.85	31.11
0.628	0.615	0.637	0.631	0.561	_	30.23	31.78	30.23
0.689	0.697	0.748	0.741	0.674	0.69		83.69	65.96
0.689	0.682	0.741	0.733	0.674	0.674	0.162		66.67
0.689	0.705	0.741	0.733	0.681	0.69	0.338	0.331	_

homologous modules of C6–C9 and perforins. Identity (*I*) between a given pair of sequences was calculated by substituting into $I = 1 - P \times 100$, where *P* is the number of differences (*P* distance) obtained using MEGA (Kumar et al. 1994). To estimate if the individual modules have diverged at different rates, the means and standard deviations were calculated from the identity values of individual modules. The statistical significance of differences in identity values among modules was tested using the Wilcoxon two-sample test included in the SAS Institute package (SAS Institute, 1985), at a 5% significance level.

Results

Phylogenetic Analysis. All C6, C7, C8α, C8β, and C9 share thrombospondin, low-density lipoprotein receptor, cysteine-poor, and epidermal growth factor modules, with a total of 444 positions, including 314 informative positions (Fig. 1). Perforins share part of the cysteinepoor and the complete epidermal growth factor modules, involving 168 positions that can be reliably aligned to C6–C9, including 133 informative positions. Identity values from pairwise comparison of sequences range between 23.66 and 87.91 (Table 1). The branch-and-bound parsimony analysis resulted in a tree showing C8a and C8ß grouped and segregated from all seven C9, C6, and C7 (Fig. 2). The internal arrangement of the C9 group, with the exception of horse C9, is in good agreement with the well-known phylogeny of these species. However, there was no resolution for the order of emergence of TCC components, as all C6-C9 appear to emerge at the base of the branch of the complement proteins. In contrast, a neighbor-joining analysis using PAUP resulted in a fully resolved tree with bootstrap values better than those obtained by parsimony phylogenetic analysis (Fig. 3). In this tree, the clade formed by C6 and C7 is placed as the sister group of the rest of the TCC components. The only anomaly in the arrangement of C9 sequences is the placement of horse C9, which also occurred in the parsimony analysis. Distance values obtained in this analysis are also shown in Table 1. One explanation for the lack of resolution of the parsimony analysis is related to the mosaic structure of C6–C9 genes; if the modules in the different proteins change at different rates, the definition of some relationships could be hindered in a parsimony analysis.

Rate of Evolutionary Change of TCC Protein Genes. To determine the rate of evolutionary change within the complete sequence data used for the analysis, a relative rate test was carried out using all pairwise comparisons among proteins (Fig. 4). Each point on the graph represents the Z value obtained by comparing the distances between a given pair of TCC proteins and perforins. Two groups of change trends were observed: C8B proteins group with all C9 except human (Fig. 4A), whereas human and rabbit $C8\alpha$ group with C6 and C7, which follow an opposite rate trend (Fig. 4C). A significant rate divergence (at the 5% confidence level) was observed only when human C9 was compared with C6 and C7 (Figs. 4B and C), however, divergences close to the cutoff value of 1.96 were also observed between human C9 and both $C8\alpha$ (Fig. 4C). Most of the other pairwise comparisons with human C9 resulted in Z values higher than the rest (Fig. 4A and B). Additional relative rate tests carried out on pairwise comparisons of individual cysteine-poor or epidermal growth factor modules indicated that significant deviations of the rate constancy were caused by the cysteine-poor module (not shown). This divergence in the change rates most likely explains the lack of resolution of the parsimony phylogenetic analysis, which does not consider heterogeneity in evolutionary rates.

	בסבים הססום המתחודה בת - ב- בי היו היהוריה ההההיה בההמת היהורה בפרותה בה 19 19 המתחודת בתבתבה בי ביו בי בי ביו	
C9puffer	CVWSRWAPWSSCDPCTNTRRRSRGVEVFGQFAGIACQGSVGDREYCITNAKCNLPPPRECSDSEFQC.ESGSCIKLRLKCNGDYDCEDGS.DE.D.CEPLRKT.CPPTVLDTN	144
C9trout	CV WSRWSEWTPCNSCTKIRHRSRSVEVFGQFGGKPCQG.QPIGEQQRCTSDAVCEQALPSECSSIEFTC.BSGAGIKLRLSCNGDYDCEDGS.DE.D.CEPVRKP.CGTKLYDTN	127
C9rat	CRM <u>5</u> TW5QW5QCDPCLKQRFRS <u>RSM</u> EV <u>FG</u> QFQGKSCA DALGDRQHCEPTQECEEVQEN. C.GNDFQC. ETGRCIKRKLLCNGDNDCGDFS. DESD. CESDP. RLP. CRDRVVEES	161
C9mus	CRM <u>SPWSNWS</u> ECDPCLKQRFRS <u>RSI</u> LA <u>FGQ</u> FNGKSCV DVLGDRQGCETTQECEEIQEN.C.GNDFQC.E <u>TGRCI</u> KR <u>RLLCNGDN</u> DCGDYS.DEND.CDDDP.RTP.CRDRVAEES	128
C9rabb	CRM <u>SPWSEWSHCDPCLRQMFRSRSI</u> EV <u>FGQ</u> FHGKSCV DALGDRRACIPTEACEDAEE . DCEKDEFHCG . <u>TGRCIKRRLLCNGDN</u> DCGDFS . DEDD . CETEP . RLT . CRNREVQES	151
C9horse	CRM <u>SSWSEWS</u> ECDPCLRQMFRS <u>RSI</u> EV <u>FGQ</u> FNGQRCV DAVGDRRQCVPTEACEEVED.DC.GNDFQCG. <u>T</u> GRC <u>I</u> KK <u>R</u> LLC NG<u>DN</u>DCGDFS.DEDD.CENDP.RPP.CRERVVEES	150
C9human	CRM <u>S</u> PWSEWSQCDPCLRQMFRS <u>RSI</u> EV <u>FGQ</u> FNGKRCT DAVGDRRQCVPTEPCEDAED.DC.GNDFQCS. <u>TGRCIKMRLRCNGDN</u> DCGDF S .DEDD.CESEP.RPP.CRDRVVEES	150
C8arabb	CQL <u>SSWSEWTDCFPCQDTKYRHRSLLQPN</u> KFGGTICSG.DIW.DRASCYSPTACLRPAQ C.GQDFQCKETGRCLKRHLVCNGENDCLDGS.DEDN.CEDIRATESDCAQYD PI	146
C8ahuman	COL SNWSEWTDCPPCODKKYRHRSLLOPNKFGGTICSG. DIW. DQASCSSSTTCVRQAQ C.GQDPQCKETGRCLKRHLVCNDQOCLDGS. DEDD. CEDVRAIDEDCSQYE . PI	146
CSbrabb	CELSSWSSWIMCDPCQKKKYRHA <u>XILLPSC</u> FNGEPCNFSKEVED.CCXISKPCRSQVR.CEG.FVCAQTGCCVRRLLCRGDDCC5QS.DEAN.CRKIYKKCHHEMKQYW	170
Conuman	CEL <u>55WB5WT1CDPCQRARKIKIATLAUPSQ</u> HHBECCNFDJACKEDCVTINKPCGQVXCEGFVCAVTGKC <u>VNKLLCNGDN</u> OCGDQB.DEAN.CKXIIACQHEMUQIW AIICDEPUNGD ADDATEVCQVIDQVI DEOROFCOATEDIVADAD. CIDOVI QVI QEDAQ VVIQEDOL ODDATEDIVAGNO CODVAV. CADVVVI DI	107
C7human	CONDITION DE L'UNITATION DE L	132
perfomus		190
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	כוסכס מי מהמני מרגיע המסמטמטמט נו מינוע איניגע מי מי מכוסס מי גווע מי מסג מאוגנוע נו ממטמטמט איניגע וווע מינוע מ	
C9puffer	EQGRTA GYGINIL. GADPRHNPPND. FFNG. RCDRVRNNNTLOLDRLEWNIGVLNYGLTNI. KNRSPMRVKGRLOLSTYRNRSHO. LOVADEFVAHVKSLPLEYERGI. YYAFLE. D	324
Corrout	ECORTA. GIGINI. GMEERINEEND. IFNG. MCNRVKNINNNETNRLEWNVGLENINTII. KANSFMKYNGRYDLSITKMRSRD. LOVAGEFLENVKSLELEITEKGO. IFSELE. D	311
Corne	ELGKIA. (YGUNLL.GHDPLAIPPLAIPPLAIPPLAIPPLAIPPLAIDELTIKKEWWARLAIDILGVIA.MARGNUULAKIVKKKK, YMLIIITLDDVIAALVVIAALV	356
Carabh	ELEMAN, CULTAIL, CHIDLATDELATTERNE, YVHG LODDUNCCHTITTURE KOMNAULAVENSES KEKKELIVEGILDE CULTAILE UNICHTELDIKELITTERKE TERKE VERLE	346
C9horse	ELARTA GYGINIL GMDPLSTPPDNE, YYNG, LCDRVRDGNTLTYYRKPWNLASLAYEYSSK, KEKMFLHVKGVIOLGKFVMRSRD, VVLTTTFLDDIKALPTAYEKGE, YIAFLE, T	341
C9human	ELARTA GYGINIL. GMDPLSTFFDNE. FYNG. LCNRDRDGNTLTYYRRPWNVASLIYEYSSK. KEKMFLHVKGEIHLGRFVMRNRD. VVLTTFFVDDIKALPTTYEKGE. YFAFLE. T	341
C8arabb	PGSEK <u>A</u> ALGYNILTQEEAOSVYDARYY.GGRCETVYNGEWRHVRYDPVCER.LHHGYNEK.KYN. <u>FM</u> RIFIK <u>V</u> QTAHFKMRRDD.IVLDEG <u>ML</u> QALVELPEQYNYGM.YSKFIN.D	336
C8ahuman	PGSQKAALGYNILTQEDAQSVYDASYY.GGQCETVYNGEWRELRYDSTCER.LYYGYNEK.KFI.FTRIFTKVQTAHFKMRKDD.IMLDEGMLQSLMELPDQYNYGM.YAKFIN.D	~ ~ ~
		336
C8brabb	AIGSLASGIN_LFMSIEG.PULDHRYYAGG.CNPHYILDMRFSHT.KSK.FLHARSALEVAHYKL.KPRNLMLHYDFLORVORVPLEYSYGE.YRD.LFRD	336 339
C8brabb C8bhuman	AIGSLASGIN_LPTNSLEG.PULDHRYYAGG.CNPHYILDMRFSHT.KSK.PLHARSALEVAHYKL.KPRNLMLHYDPLORVORVPLEYSYGE.YRD.LFRD GIGSLASGIN_LPTNSPEG.PULDHRYYAGG.CSPHYILNTRFSHT.KSV.PLHARSDLEVAHYKL.KPRSLMLHYEPLORVKRLPLEYSYGE.YRD.LPRD	336 339 339
C8brabb C8bhuman C6human	AIGSLASGIN_LFTNSLEG.PULDHRYYAGG.CNPHYILDMRFSHT.KSK.PLHARSALEVAHYKL.KPRNLMLHYDPLORVORVPLEYSYGE.YRD.LFRD GIGSLASGIN_LFTNSPEG.PULDHRYYAGG.CSPHYILNTRFSHT.KSV.FLHARSDLEVAHYKL.KPRSLMLHYEPLORVKRLPLEYSYGE.YRD.LFRD PSVQLMGNOFHPLAGE.PEGEVLDNS.FT.GGICKTVKSSRTSNPYRVPANLEVTVASHK.KDSSFIRIHKVMKVLAFTT.KAKDLHLSDVFLKALNHLPLEYN.SALYSR.IFDD	336 339 339 360
C8brabb C8bhuman C6human C7human	AIGSLASGIN_LFTNSLEG.PULDHRYVAGG.CNPHYILDMRFSHT.KSK.FLHARSALEVAHYKL.KPRNLMLHYDFLQRVQRVPLEYSYGE.YRD.LFRD GIGSLASGIN_LFTNSEG.PULDHRYVAGG.CSPHYILNTRFSHT.KSV.FLHARSDLEVAHYKL.KPRSLMLHYBFLQVXKLPLEYSYG.YRD.LFRD PSVQLM.GNGYHELGGE.PGGEVLDNS.FT.GGICKTVKSSRTSNPYRVPANLEWNASKK.KDSSFINHKYMKULHFT.KAKDHLSDVFLKALMHLPLEYN.SALYSR.IFDD PNIELT.GNGYNELTGQFYNR.VINTKSFGGQCRKVFSGDGKDFYRLSGN.VLSYT.HKGKSYQLLVVENTVEVAQFINNNPEFLQLAEPFMKELSHLPSLYDYSA.YRR.LIQ VYP YYC	336 339 339 360 296
C8brabb C8bhuman C6human C7human perfomus perforat	AIGSLASGIN_LFTNSTEG, PULDHRYYAGG. CNPHYILDMRFSHT.KSK.FLHARSALEVAHYKL.KPRNLMLHYDFLQRVQRVPLEYSYGE.YRD.LFRD GIGSLASGIN_LFTNSFEG.PULDHRYYAGG.CSPHYILNTRFSHT.KSV.FLHARSDLEVAHYKL.KPRSLMLHYEFLQRVKHLPLEYSYGE.YRD.LFRD PSVQLM.GNGPHELAGE.PRGEVLDNS.FT.GGICKTVKSSFTSNPYRVPANLENVASHK.KDSSFILHKVMKVLHFT.KKALLESVFLKALMLPLEYSYG.SALYSR.IFDD PNIELT.GNGYNELIGQFVNR.VINTKSF.GGQCRKVFSGDGKDFYRLSGN.VLSYT.HKGKSYQLLVVENTVEVAQFINNNPEFLQLAEPFWKELSHLPSLYDYGA.YRR.LIDQ YRR.LISS	336 339 339 360 296 217 217
C8brabb C8bhuman C6human C7human perfomus perforat perfohum	AIGSLASGIN_LFTNSTEG.PULDHRYYAGG.CNPHYILDMRFSHT.KSK.PLHARSALEVAHYKL.KPRNLMLHYDFLQRVQRVPLEYSYGE.YRD.LFRD GIGSLASGIN_LFTNSTEG.PYLDHRYYAGG.CSPHYILNTRFSHT.KSV.PLHARSDLEVAHYKL.KPRSLMLHYEFLQRVKHLPLEYSYGE.YRD.LFRD PSVQLMGNGFHFLAGE.PRGEVLDNS.FT.GGICKTVKSSRTSNPYRVPANLENVASHK.KDSSFIRIHKVMKVLNFTT.KAKDLHLSOVFLKALNHLPLEYN_SALYSR.IFDD PNIELT.GNGYNELTGQFVNR.VINTKSF.GGOCRKVFSGDGKDFYRLSGN.VLSYT.HKGKSYOLLVVENTVEVAQFINNNPEFLQLAEPFWKELSHLPSIYDSA.YRR.LISS YRR.LISS YRR.LISS YRR.LISS	336 339 339 360 296 217 217 217
C8brabb C8bhuman C6human C7human perfomus perforat perfohum	AIGSLASGIN_LFTNSTEG, <u>PULDHRYYAGG</u> .CNPHYILDMRFSHT.KSK. <u>PLHARSAT</u> EVAHYKL.KPRNLMLHYD <u>FLORVORV</u> PLEYSYGE.YRD.LFRD GIGSLASGIN_LFTNSTEG, <u>PULDHRYYAGG</u> .CSPHYILNTRFSHT.KSV. <u>PLHARSDLEVAHYKL.KPRSLMLHYEFLORVKRLPLEYSYGE</u> .YRD.LFRD PSVOLMGNGYNELTGOFVLDNS.FT.GGICKTVKSSRTSNPYRVPANLENVASHK.KDSSFIRIHKVMKVLNFTT.KAKDLHLSDVFLKALNHLPLEYNSALYSR.IFDD PNIELT.GNGYNELTGOFVNR. <u>VI</u> NTKSF.GGOCRKVFSGDGKDFYRLSGN.VLSYT.HKGKSYQLLVYENTYEVAQFINNNPEFLOLAEPFWKELSHLPSLYDYSA.YRR.LIDS YRR.LISS YRR.LISS	336 339 339 360 296 217 217 217
C8brabb C8bhuman C6human C7human perfomus perforat perfohum	AIGSLASGIN_LFTNSLEG, PULDHRYYAGG CNPHYILDMRFSHT.KSK.FLHARSALEVAHYKL.KPRNLMLHYDFLQRVQRVPLEYSYGE.YRD.LFRD GIGSLASGIN_LFTNSFEG.PULDHRYYAGG CSPHYILNTRFSHT.KSV.FLHARSDLEVAHYKL.KPRSLMLHYEFLQRVKHLPLEYSYGE.YRD.LFRD PSVQLM.GNGPHFLAGE.PEGEVLDNS.FT.GGICKTVKSSRTSNPYRVPANLENVASHK.KDSSFLHKVMKVLHFT.KAKDLEUQAVKHLPLEYN.SALYSR.IFDD PNIELT.GNGYNELTGQFVNR.VINTKSFGGQCRKVFSGDGKDFYRLSGNVLSYT.HKGKSYQLLVVENTVEVAQFINNNPEFLQLAEPFMKELSHLPSLYDYSA.YRR.LIDQ YMR.LISS YRR.LISS YRR.LISS	336 339 339 360 296 217 217 217
C8brabb C8bhuman C6human C7human perfomus perforat perfohum	AIGSLASGIN_LFTNSTEG, PULDHRYYAGG CNPHYILDMRFSHT.KSK.FLHÄRSALEVAHYKL.KPRNLMLHYDFLORVQRVPLEYSYGE.YRD.LFRD GIGSLASGIN_LFTNSFEG.PULDHRYYAGG CSPHYILNTRFSHT.KSV.FLHARSDLEVAHYKL.KPRSLMLHYEFLORVKRLFLEYSGE.YRD.LFRD PSVOLM.GNGPHELAGE.PRGEVLDNS.FT.GGICKTVKSSRTSNPYRVPANLENVASHK.KDSSFLHKHYKNLKLHFLSVFLKALMLPLEYS.SLYSR.IFDD PNIELT.GNGYNELTGOFVNR.VINTKSF.GGOCRKVFSGDGKDFYRLSGN.VLSYT.HKGKSYOLLVVENTVEVAQFINNNPEFLOLAEPFWKELSHLPSLYDYSA.YRR.LIDQ YRR.LISS YRR.LISS YRR.LISS	336 339 360 296 217 217 217
C8brabb C8bhuman C6human C7human perfomus perforat perfohum	AIGSLASGIN_LFTNSTEG.PULDHRYYAGG.CNPHYILDMRFSHT.KSK.PLHÄRSALEVAHYKL.KPRNLMLHYDPLORVORVPLEYSYGE.YRD.LFRD GIGSLASGIN_LFTNSTEG.PYLDHRYYAGG.CSPHYILNTRFSHT.KSV.PLHARSDLEVAHYKL.KPRSLMLHYEFLORVKLPLEYSYGE.YRD.LFRD PSVOLMGNGFHFLAGE.PRGEVLDNS.FT.GGICKTVKSSRTSNPYRVPANLEVNASKK.KDSSFIRIHKVMKVLNFTT.KAKDLHLSDVFLKALNHLPLEYSYGE.YRD.LFRD PNIELTGNGYNELTGOFYNR.VINTKSFGGOCRVVFGDSKDFYRLSGN.VLSYT.HKGKSYOLLVVENTVEVAOFINNNPEFLOLAEPYKKELSHLPSLYDYSA.YRR.LIDO YHR.LISS YRR.LISS YRR.LISS	336 339 360 296 217 217 217
C8brabb C8bhuman C6human C7human perfomus perforat perfohum C9puffer	AIGSLĀS GĪN_LFŢNSĪĒG, PŪLDHRYVAGG CNPHYILDMR	336 339 360 296 217 217 217
C8brabb C8bhuman C6human C7human perfomus perforat perfohum C9puffer C9puffer	AIGSLASGIN_LFTNSTEG, PULDHRYYAGG CNPHYILDMRFSHT.KSK.FLHÄRSALEVAHYKL.KPRNLMLHYDFLQRVQRVPLEYSYGE.YRD.LFRD GIGSLASGIN_LFTNSTEG, PULDHRYYAGG CSPHYILNTRFSHT.KSV.FLHÄRSDLEVAHYKL.KPRSLMLHYEFLQRVKRLPLEYSYGE.YRD.LFRD PSVQLM.GQGPHELGG.PEGEVLDNS.FT.GGICKTVKSSRTSNPYRVPANLENVASHK.KDSSFLHKVMKVLHFTT.KAKPLHSVTGKLANHLPLEYN,SALYSR.IFDD PNIELT.GNGYNELTGQFYNR.VINTKSF.GGQCRKVFSGDGKDFYRLSGN.VLSYT.HKGKSYQLLVVENTVEVAQFINNNPEFLQLAEPFMKELSHLPSLYDYSA.YRR.LIDQ 	336 339 360 296 217 217 217 217 88
C8brabb C8bhuman C6human C7human perfomus perforat perfohum C9puffer C9puffer C9trout C9trout	AIGSLASGIN_LFTNSTEG, PULDHRYYAGG CNPHYILDMRFSHT.KSK.FLHÄRGALEVAHYKL.KPRNTMTHYDFLORVORVPLEYSYGE.YRD.LFRD GIGSLASGIN_LFTNSTEG, PULDHRYYAGG CSPHYILNTRFSHT.KSV.FLHÄRGDLEVAHYKL.KPRSTMTHYDFLORVKLFTS, SALYSR.IFDD PNOLM.GNGEHELGGE PRGEVLONS.FT.GGICKTVKSSRTSTPYRVPANLENVASHK.KDSSFILHKVMKVLHFTT.KKALLEDVFLKALMHLPLEYN SALYSR.IFDD PNIELTGNGYNELTGOFVNR.VINTKSF.GGOCKVFSGDGKDFYRLSGN.VLSYT.HKGKSYOLLVVENTVEVAQFINNNPEFLOLAEPFWKELSHLPSLYDYSA.YRR.LIDQ YHR.LISS YRR.LISS YRR.LISS YRR.LISS YRR.LISS YLR.LISN YGTHYTKNGKSGGEYE.LVYVINOTIKKK.LTE.RKIQCELKIGIVVDNYMTSVKGSLESAVTMMARTIASA.PALLNSEPEPIYM.LPTDIPGA.NSEINNLGATADYVAE 4 YGTHYTKNGKSGGEHC.LVYVINOKSK.LTE.RKIQCELKIGIVDNYMTSVKGGSLESAVTMARTIASA.PALLSSEPEPIYM.LPIDIPGA.NSEINNTKLMQATQEYEAE	336 339 360 296 217 217 217 217 88 75 26
C8brabb C8bhuman C6human C7human perfomus perforat perfohum C9puffer C9puffer C9trout C9rat C9rat	AIGSLĀS GĪN_LFŢNSĪĒG, PŪLDHRYVAGG CNPHYILDMR	336 339 339 296 217 217 217 217 88 75 26 87
C8brabb C8bhuman C6human C7human perfomus perforat perfohum C9puffer C9trout C9rat C9mus C9mus C9rabb	AIGSLASGIN_LFTNSTEG. PULDHRYYAGG.CNPHYILDMRFSHT.KSK.FLHÄRSALEVAHYKL.KPRNTMIHYDFLQRVQRVPLEYSYGE.YRD.LFRD GIGSLASGIN_LFTNSTEG.PULDHRYYAGG.CSPHYILNTRFSHT.KSV.FLHÄRSDLEVAHYKL.KPRSTMIHYETQAVKIPLEYSYGE.YRD.LFRD PSVQLM.GQGPHELGG.PCGEVLDNS.FT.GGICKTVKSSRTSNPYRVPANLENVASKK.KDSSFIRIHKVMKULHFTT.KAKDLENDETQAVKHTPLEYSYGE.YRD.LFRD PNIELT.GNGYNELTGQFYNR.VINTKSFGGQCRKVFSGDGKDFYRLSGN.VLSYT.HKGKSYQLLVVENTVEVAQFINNNPEFLQLAEPFMKELSHLPSLYDYSA.YRR.LIDQ YRR.LISS YRR.LISS YLR.LISS YLR.LISS YLR.LISS YLR.LISS YGTHYTKNGKSGGEFE.LVV.VINQDTIKAKN.LTE.RKIGECIKIGIVUDNVTSVKGGSLESAVTMMARTYGDA.PALLSSEPEFLYN.LIPTDIPGA.NSFIANLKQATADYVAE 4 YGTHYTKNGKSGGEFE.LVV.VINQDTIKAKN.LTE.RKIGECIKIGIVUDNVTSVKGGSLESAVTMMARTYGDA.PALLSSEPEFLYN.LIPTDIPGA.NSFIANLKQATADYVAE 4 YGTHYTSSGSLGGUYE.LIY.VINQDTIKKK.GVE.LSDVKCL.GFIIDDVISFIRGGTRKQAVLMASSLDA.PALLSSEPEFLYN.LIPLSMPA.YKKQMBEKAEDVYNE 5 YGTHYSSGSLGGUYE.LIY.VILDKASMKEK.GVE.LNDVKKCL.GFIIDDVISFIRGGTRKQAVLLMASSLDA.PALLSQKJEFIYN.LIPLKKKDA.YKKQMLEKAZVEDVIDE 4	336 339 360 296 217 217 217 88 75 26 87 14
C&brabb C&bhuman Cfhuman perfomus perfonus perfohum C9puffer C9trout C9trout C9rat C9mus C9rabb C9horse	AIGSLASGIN_LFTNSTEG, PULDHRYYAGG CNPHYILDMRFSHT.KSK.FLHÄRSALEVAHYKL.KPRNLMLHYDFLQRVQRVPLEYSYGE.YRD.LFRD GIGSLASGIN_LFTNSTEG, PULDHRYYAGG CSPHYILNTRFSHT.KSV.FLHÄRSDLEVAHYKL.KPRSLMLHYELQRVKHLALNHVALSV, GNOEHLAGE PEGEVLONS.FT.GGICKTVKSSTSTPYRVPANLENVASKK.KDSSFIRINKWKVLHFT.KKNGLHSVFLKALMHUPLEYN.SALYSR.IFDD PNIELTGNGYNELTGQFYNR.YINTKSFGGQCRKVFSGDGKDFYRLSGN.VLSYT.HKGKSYQLLVYENTYEVAQFINNNPEFLQLAEPFMKELSHLPSLYDYSA.YRR.LIDQ YRR.LISS YLR.LISS YLR.LISS YLR.LISS YLR.LISS YLR.LISS YGTHYTKNGKSGGEFY.LVY.VINOTIKKK.LTE.RKIQCELKIGIVVDNYMTSVKGGLESAVTMARTIASA.PALINSEPEPIYM.LIPTDIPGA.NERIANLKAATADVAE 4 YGTHYTKNGKSGGEFY.LIYY.VINOTIKKK.LTE.RKIQCELKIGIVVDNYMTSVKGGLESAVTMARTIASA.PALINSEPEPIYM.LIPTDIPGA.NERIANLKAATADVAE YGTHYTKNGKSGGEFY.LIYY.VILAGNTKKK.GVE.LSDYKRCLGFIIDDYISFIRGGTRKQAVLLMASSLDA.PALISSPEPIYN.LIPINTMAA.YAKKQMMEKAIEDVYNE 5 YGTHYTSSGSLGGYY.LIYY.VILAGMKEK.GVE.LSDYKRCLGFIIDDYISFIRGGTRKQAVLLMASSLDA.PALISSPEPIYN.LIPITMKDA.YAKKQNMEKAIEDVYNE 5 YGTHYSSGSLGGYYE.LIY.VILAGMKEK.GVE.LSDYKRCLGFIIDDYISFIRGGTRKQAVLLMASSLDA.PALISSPEPIYN.LIPITMKDA.YAKKQNEKAIEDVYNE 5 YGTHYSSGSLGGYYE.LIY.VILAGMKEK.GVE.LSDYKRCLGFIIDDYISFIRGGTRKQAVLLMASSLDA.PALISSPEPIYN.LIPITMKDA.YAKKONLEKAYEDVINE 5 YGTHYSSGSLGGYYE.LIY.VILAGMKEK.GVE.LNDIKKCLGFIIDDYISFIRGGTRKAALLMASSLDA.PALISSPEPIYN.LIPVKKNA.HKKONLEKAYEDVINE 5 YGTHYSSGSLGGYYE.LIY.VILAGMKEK.GVE.LNDIKKCLGFIIDDYISFIRGTRKAALLMASSLDA.PALISORASFIYN.LIPVKKAA.HKKKANEKEGIDYNE 5 YGTHYSSSGSLGGYYE.LIY.VILAKSMKEK.GVE.LNDIKKEL.GFIIDDYISFIRGTRKAALLMASSLDA.PALISORASFIYN.LIPVKKAA.HKKKANEKEGINIF	336 339 339 296 217 217 217 217 88 75 26 87 14 04
C8brabb C8bhuman C6human C7human perforat perforat perforat C9puffer C9trout C9rat C9rab C9rabb C9rabb C9human	AIGSLASGIN_LFTNSTEG, PULDHRYYAGG CNPHYILDMRFSHT.KSK.FLHÄRSALEVAHYKL.KPRNTMLHYDFLORVORVPLEYSYGE.YRD.LFRD GIGSLASGIN_LFTNSTEG, PULDHRYYAGG CSPHYILNTRFSHT.KSV.FLHÄRSDLEVAHYKL.KPRSTMLHYEFLORVORVELEYSYGE.YRD.LFRD GIGSLASGIN_LFTNSTEG, PULDHRYYAGG CSPHYILNTRFSHT.KSV.FLHÄRSDLEVAHYKL.KPRSTMLHYEFLORVORVELESYGE.YRD.LFRD GIGSLASGIN_LFTNSTEG, PULDHRYYAGG CSPHYILNTRFSHT.KSV.FLHÄRSDLEVAHYKL.KPRSTMLHYEFLORVORVELESYGE.YRD.LFRD GIGSLASGIN_LFTNSTEG, PULDHRYYAGG CSPHYILNTRFSHT.KSV.FLHÄRSDLEVAHYKL.KPRSTMLHYEFLORVORVELESYGE.YRD.LFRD PNIELT.GNGYNELTGOFYNR.VINTKSF.GGOCRKVFSGDGKDFYRLSGN.VLSYT.HKGKSYOLLVYENTYEVAQFINNNPEFLOLAEPFWKELSHLPSLYDYSA.YRR.LIDO YRR.LISS YLR.LISS YLR.LISS YLR.LISS YGTHYTKNGKSGGEYE.LVY.JUNODTIKAKN.LTE.RKIQCELKIGIVUDNYMTSVKGGSLESAVTMWARTIASA.PALLSSEPEPIYM.LIPTDICA.NSRIANLKOATADYVAE 4 YGTHYSSGSLGGUYE.LIY.YUNONTIKKK.LTE.RKIQCELKIGIVDNYMTSVKGGSLESAVTMARTIASA.PALLSSEPEPIYM.LIPTDICA.NSRIANLKOATADYVAE 4 YGTHYSSGSLGGUYE.LIY.YULDKASMKEK.GVE LSDYKKCL.GFIIDDYISFIRGTRKQAVLLMASSIDDA.PALLSSEPEPIYM.LIPLNKAA.YAKKONMEKALBOVINE 5 YGTHYSSGSLGGUYE.LIY.YULDKASMKEK.GVE LSDYKKCL.GFIIDDYISFIRGTRRQAVLLMASSIDA.PALLSOKLSPIYM.LIPLKKAA.YAKKONMEKALBOVINE 5 YGTHYSSGSLGGUYE.LIY.YULDKASMKEK.GVE LKDICKCL.GFIIDDYISFIRGTRRQAVLLMASSIDA.PALLSOKLSPIYM.LIPLKKAA.YAKKONMEKALBOVINE 5 YGTHYSSGSLGGUYE.LIY.YULDKASMKEK.GVE LKDICKCL.GFIIDDYISFIRGTRRQAVLMASSIDA.PALLSOKLSPIYM.LIPLKKAA.YAKKONMEKALBOVINE 5 YGTHYSSGSLGGUYE.LIY.YULDKASMKEK.GVE LKDICKCL.GFIIDDYISFIRGTRRQAVLMASSIDA.PULNOKLSPIYM.LIPVKKAA.HKKAAMEKARGEDYINE 5 YGTHYSSGSLGGUYE.LIY.YULDKASMKEK.GVE LKDICKCL.GFIIDDYISFIRGTRRQAVLMASSIDA.PULNOKLSPIYM.LIPVKKAA.HKKAAMEKARGEDYINE 5 YGTHYSSGSLGGUYE.LIY.YULDKASMKEK.GVE LKDICKCL.GFIIDDYISFIRGTRRAATLYAFELMASSIDA.PULNOKLSPIYM.LIPVKKAA.HKKAAMEKARGEDYINE 5 YGTHYSSGSLGGUYE.LIY.YULDKASMKEK.GVE LKDICKCL.GFIIDDYISLIRGTRKYAFELMASSINDA.PULNOKLSPIYM.LIPVKKAA.HKKAA.HKKAAFARIBDYINE 5	336 339 339 296 217 217 217 88 75 26 87 14 04
C&brabb C&bhuman C&human perforat perforat perfohum C9puffer C9trout C9rat C9rab C9horse C9horse C9human C8arabb	AIGSLAS GIN_LFTNSTEG, PULDHRYNAGG CNPHYILDMR	336 339 360 296 217 217 217 217 88 87 526 87 14 04 95 93
C&brabb C&bhuman C&human Crhuman perforus perforus perfohum C9puffer C9trout C9rat C9rat C9rab C9horse C9human C&arabb C&ahuman C&brabb	AIGSLASGIN_LFTNSTEG, PULDHRYYAGG CNPHYILDMR	336 339 360 296 217 217 217 217 88 87 26 87 14 04 95 93 99
C&brabb C&bhuman C6human perfomus perforat perfohum C9puffer C9trout C9trout C9rab C9rabb C9horse C9horse C9human C&ahuman C&bhuman	AIGSLASGIN_LFTNSTEG, PULDHRYYAGG CNPHYILDMRFSHT.KSK.FLHÄRSALEVAHYKL.KPNNLMLHYDFLQRVQRVPLEYSYGE.YRD.LFRD GIGSLASGIN_LFTNSTEG, PULDHRYYAGG CSPHYILNTRFSHT.KSK.FLHÄRSDLEVAHYKL.KPNSLMLHYEFLQRVKRLPLEYSYGE.YRD.LFRD GIGSLASGIN_LFTNSTEG, PULDHRYYAGG CSPHYILNTR	336 339 360 296 217 217 217 88 75 26 87 14 04 99 99 99 99
C&brabb C&bhuman C&human Crhuman perforat perforat perfohum C9puffer C9trout C9rat C9rab C9horse C9human C&brabb C&bhuman C&brabb C&bhuman	AIGSLAS GIN_LFTNSTEG, PULDHRYYAGG CNPHYILDMR	336 339 360 296 217 217 217 88 75 26 87 26 87 26 93 99 99 917
C&brabb C&bhuman C&human perforus perforus perfohum C9puffer C9trout C9rat C9rat C9mus C9rabb C9horse C9human C&brabb C&bhuman C&bhuman	AIGSLASGIN_LFTNSTEG, PULDHRYYAGG CNPHYILDMR	336 339 360 217 217 88 75 287 14 04 99 99 917 47
C&brabb C&bhuman C6human C7human perfomus perfohum C9puffer C9trout C9rat C9rat C9rab C9horse C9horse C9horse C9horse C9human C&brabb C&bhuman C8brabb	AIGSLASGIN_LFTNSTEG, PULDHRYYAGG CNPHYILDMRFSHT.KSK.FLHÄRSALEVAHYKL.KPNIM_HYDFLQRVQRVPLEYSYGE.YRD.LFRD GIGSLASGIN_LFTNSTEG, PULDHRYYAGG CSPHYILNTRFSHT.KSV.FLHÄRSDLEVAHYKL.KPRSIM_HYBFLQRVKKLPLEYSYGE.YRD.LFRD GIGSLASGIN_LFTNSTEG, PULDHRYYAGG CSPHYILNTR	336 339 3296 2177 217 88 75 287 14 99 99 917 47 59
C8brabb C8bhuman C6human perforus perforus perforus c9trout C9trout C9trout C9trout C9rabb C9horse C9horse C9horse C9horse C8ahuman C8brabb C8bhuman C6buman c7human perforus perforus	AIGSLAS GIN_LFTNS_EG. EVLDHRYYAGG.CNPHYILDMR	336 3399 3360 2217 217 88 75 687 14 04 95 999 917 17 599 9999 177 559
C&brabb C&bhuman C&human Cohuman perforat perforat perfohum C9puffer C9trout C9rat C9rat C9rabb C9horse C9human C&brabb C8bhuman C&brabb C8bhuman C6human perfomus perfonus perfonus	AIGSLGSGIN_LFTNS_EG. <u>F</u> ULDHRYYAGG.CNPHYILDMR	336 339 3360 2217 217 88 75 26 87 2217 88 75 26 87 14 04 99 99 99 99 99 99 99 99 99 77 75 59 99 99 99 77 55 99 99 99 77 75 55 99

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		0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0		Excluded sites
C9puffer	YNVCKCRPCHNGGTLALLDGKCICMCSNLFEGLG.CQWSCWSSWSNCQ.GQKRSRTRYC	NTEGVL GAECRGEIRSEEYC	586 l	-36, 200-268, 369-404, 425-441, 525-545
C9trout	YSVCKCKPCHNGGSLALLDGKCLCLCLPQFEGLA.CQWSCWAAWSGCS.GGKRIRTRSC	MTQGLS DATCRGDIVTEDYC	574 1	-18, 183-255, 356-391, 412-428, 512-533
C9rat	FSARKCYPCQNGGTAILLDGQCMCSCTIKFKGIA.CE		562 1	-53, 217-300, 399-441, 462-479, 563-567
C9mus	FSTKRCYPCLNGGTIILLDGQCLCSCPMMFRGMA.CE		523 1	-20, 182-263, 362-402, 423-440, 524-528
C9rabb	FSTKKCSPCQNGGTALLMDGQCLCTCPFMFEGIA.CE		550 l	-42, 207-290, 389-429, 450-467, 551-557
C9horse	FSVRKCHPCQNGGTVIQIDGQCLCSCPIAFEGIA.CE		540 1	-42, 206-285, 384-419, 440-457, 541-547
C9human	FSVRKCHTCQNGGTVILMDGKCLCACPFKFEGIA.CE		540 1	-42, 206-285, 384-420, 441-457, 541-559
C8arabb	FNACRCGPCFNNGKPILEGTSCRCQCSLGLQGPA.CEWSCWGSWSPCTAGTRERRRE.C	<u>NN</u> PAPQNGGAPCPGWRVQTQAC	585 l	-38, 201-281, 380-417, 436-446, 531-542
C8ahuman	FNACRCGPCFNNGVPILEGTSCRCQCRLGSLGAA.CEWSCWSSVCRAGIQERRRE.C	DNPAPQNGGASCPGRKVQTQAC	584 1	-38, 201-281, 380-417, 435-445, 530-541
C8brabb	VSPCRCAPCQGNGVPVQKGSRCDCICPVGFQGSA.CEWSCWSRWSSCSGGQKTRRRQ.C	<u>NN</u> PAPQDGGSPCSGPASETLAC	590 1	-64, 210-284, 384-421, 439-451, 536-547
C8bhuman	VSSCHCAPCQGNGVPVLKGSRCDCICPVGSQGLA.CEWNCWSNWSSCSGRRKTRQRQ.C	<u>NN</u> PPPQNGGSPCSGPASETLDC	590 1	-64, 210-284, 384-421, 439-451, 536-547
C6human	FDPCQCAPCPNNGRPTLSGTECLCVCQSGTYGEN.CEWGCWSSWSTCDATYKRSRTREC	NNPAPQRGGKRCEGEKRQEEDC	611 l	-81, 240-304, 405-442, 456-471, 554-567, 612-934
C7human	. DP CHCRPCQNGGLATVEGTHCLCHCKPYTFGAA . CEWSCWSSWSPCVQGKKTRSR . EC	<u>NN</u> PPPSGGGRSCVGETTESTQC	543 1	-27, 188-242, 343-376, 389-405, 488-502, 546-843
perfomus	RARWQNCS.RPCRS.GQHKSSHDSCQCECQDSKVTNQDC		406 1	-210, 262-302, 314-326, 407-554
perforat	RARWRDCN.RPCRA.GQHKSSRDSCQCVCQDSNVTNQDC		406 1	-210, 262-302, 314-326, 407-554
perfohum	RARWRDCS.RPCPP.GRQKSPRDPCQCVCHGSAVTTQDC		406 1	-211, 263-303, 315-327, 406-555
				and the second

Bold = Identical positions Underlined = Same group amino acids = Informative position



Points where variable sites were excluded
Thrombospondin module
Low Density Lipoprotein receptor module

------ = Cysteine-poor region
 ------- =Epidermal Growth Factor module

Fig. 1. Alignment of partial amino acid sequences of C6–C9 and performs used for the phylogenetic analyses. All sites for which positional homology was uncertain were excluded (listed at the end of the alignment). Numbering of sequences is used as they appear in

GenBank. puffer, puffer fish; trout, rainbow trout; mus, mouse; rabb, rabbit; perfomus, mouse perforin; perforat, rat perforin; perfohum, human perforin.

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Fig. 2. Parsimony analysis. Fifty percent majority-rule consensus tree resulting from a branch-and-bound analysis of the amino acid sequences of C6–C9 with 100 bootstrap replicates. Perforins were used as outgroups.

If a given module has changed at a rate significantly different from the others, its identity values should correlate with these differences. As shown in Table 2, identity values of the low-density lipoprotein and carboxylterminal thrombospondin modules are significantly different from those of the other modules, whereas the cysteine-poor and amino-terminal thrombospondin identity values are not significantly different from that of the epidermal growth factor module. The mean of identity values within the cysteine-poor region is the lowest, suggesting that this module has changed more rapidly than the rest.

Discussion

Our results from the distance phylogenetic analysis using all deduced amino acid sequences available for performs and C6–C9 suggest that C6 and C7 were the earliest of the homologous TCC proteins to emerge during evolution. Performs have a simple modular composition including single cysteine-poor and epidermal growth factor modules. Among TCC components, the C9 proteins are the simplest, with cysteine-poor and epidermal growth factor modules like the performs, plus thrombospondin and low-density lipoprotein receptor modules. In addition to the cysteine-poor, epidermal growth factor, low-density lipoprotein receptor, and thrombospondin modules of C9, C8 α and C8 β possess an extra throm-

Table 2. Average pairwise identity values for individual thrombospondin, low-density lipoprotein receptor, cysteine-poor, and epidermal growth factor modules among TCC proteins

Average	SD ^a	
63.9	11.5 a	
50.1	11.5 b	
43.8	15.3 c	
42.2	16.7 cd	
38.4	16.7 d	
	Average 63.9 50.1 43.8 42.2 38.4	

^a Letters following the SD values show the results from a Wilcoxon two-sample test. Data followed by different letters are significantly different from each other (p = 0.05).

bospondin module (also present in C9 from fish). The more complex modular structures of C6 and C7 show additionally two short consensus repeats and two complement factor I modules; finally, C6 also possesses one more amino-terminal thrombospondin module (Hobart et al. 1995). The tree obtained from our distance analysis suggests that duplication of the ancestral gene with a complex modular composition evolved through two pathways, each characterized by a tendency to lose modules: one pathway led to the simple structure of perforin, which functions independently to form pores. The second evolutionary pathway produced the ancestor of C6-C7, with its complex modular structure. Further duplications and loss of modules led successively to C8 and C9. The TCC evolutionary pathway, unlike the perforin pathway, retained the evolutionary intermediate proteins (C6–C8), presumably because of their function in signaling, vide infra.

Our results suggest a single origin for C6-C9 proteins and that the minimum common ancestor of the terminal components must have possessed a complex modular structure, in agreement with the concept that it extended from the amino-terminal region of C6 up to exon 10 or 11 of the carboxyl-terminal of C8 (Hobart et al. 1993, 1995; Hobart 1998). The fact that puffer fish and trout C9's are similar in their modular composition to human rabbit C8 α and C8 β (Yeo et al. 1997; Tomlinson et al. 1993), and different from the more recent C9 of mammals, might be indicative of this tendency to lose modules after the emergence of the ancestral complex gene. Hobart et al. (1993, 1995; Hobart 1998) also proposed that C6 and C7 emerged first, then C8, and later C9. In their genetic analysis of C6 and C7 proteins, they noted that intron-exon boundaries occurred commonly within modules, as opposed to between modules. This complex intron-exon relationship between the low-density lipoprotein receptor and the cysteine-poor, as well as between the low-density lipoprotein receptor and the adjacent thrombospondin module is conserved from C6 to C9. Thus, it would be unlikely that C9 could have arisen by simple exon shuffling.

The earlier emergence of C6 and C7 has functional implications: C5b and C5b6 cannot signal, but C5b67



can transduce intracellular signals by two mechanisms (Vanguri and Shin 1988; Niculescu et al. 1993). C5b67 can insert into the membrane (Hammer et al. 1975) and signal using Gi (Niculescu et al. 1994) proteins or can refold and signal from outside the cell (Wang et al. 1995), also using Gi proteins (Wang et al. 1996). Importantly, C5b67 is the smallest functioning signaling unit of the TCC. The subsequent emergence of C8 would have allowed a floppy C5b678 channel to form (Gee et al. 1980), which enhanced the basal Gi signaling with a modest Ca²⁺ flux. Finally, the emergence of C9 would allow a rigid channel to form and a major Ca²⁺ flux could supplement the basal Gi signaling (Michaels et al. 1976). The functional consequences of the incremental signaling afforded when C8 or C8 plus C9 is added to membrane-inserted C5b-7 are well documented (Niculescu et al. 1993, 1997). Although purified C9 can polymerize by itself to form a channel in vitro, the conditions necessary for this activity are not physiologic, i.e., a high molar concentration of C9, an absence of C5b-8, and an ab-

Fig. 3. Distance analysis. Fifty percent majority-rule consensus tree resulting from a neighbor-joining analysis of the amino acid sequences of C6–C9 with 100 bootstrap replicates. Branch lengths (in *italics*) and bootstrap values are also shown. Perforins were used as outgroups.

sence of control proteins (DiScipio and Hugli 1985). In normal circumstances C8 can bind only to C5b–7, while C9 can bind only to C5b–8. This enforced order of reactivity provides further functional evidence that the order of evolution was from C6 to C9.

In summary, our phylogenetic analysis strongly supports the earlier emergence of C6 and C7, followed successively by C8 and C9. The functional similarities between C9 and perforins have two independent origins in the phylogeny presented, and the selective conditions that led to these two proteins with pore-forming ability were presumably different. The need for a cell lytic potential led to the evolution of perforin, whereas the selective advantage of enhancing the C5b–8 signal through increasing the Ca²⁺ flux led to the evolution of C9. The divergence after the duplication of the common ancestral gene, which gave rise through separate pathways to the pore-forming perforin and C9, is supported by the fact that nonhomologous regions appear to be responsible for the lytic activity in both proteins (Liu et al. 1995). Ac-



Fig. 4. Heterogeneity of evolutionary change among C6-C9. Statistic Z values obtained from the relative rate test by pairwise comparisons among sequences. Each point on the graph represents the Z value obtained from comparing the distance from a couple of sequences to the perforins. Points are joined by lines only for presentation purposes. The horizontal line shows the cutoff value (above 1.96 is considered to be out of a constant evolution rate). C9.puf, puffer fish C9; C9.tro, rainbow trout C9; C9.mus, mouse C9; C9.rat, rat C9; C9.rab, rabbit C9; C9.hor, horse C9; C9.hum, human C9; C8α.rab, rabbit C8α; C8α.hum, human C8α; C8β.rab, rabbit C8β; C8B.hum, human C8B; C6.hum, human C6; C7.hum, human C7.

cording to this view, the pore-forming ability shared by C9 and perforin is an example of parallelism.

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