16 Cysteine-rich Secretory Proteins in Reptile Venoms

William H. Heyborne and Stephen P. Mackessy

CONTENTS

Cysteine-rich secretory proteins (CRISPs) are found in a wide variety of animal tissues, particularly the epididymis of mammals, and most reptile venoms appear to contain at least one isoform. Although several venom CRISPs have been assigned specific functions, many have not, and the biological significance of this family of proteins in venoms is not clear. In many colubrid venoms, they are major protein constituents, suggesting that they have an important role in envenomation. Like many other families of reptile toxins, CRISPs show a highly conserved molecular scaffold, and the sixteen cysteines and eight disulfides they form are 100% conserved. Because they are widely distributed among reptile venoms, show structural conservation, and many have been sequenced, they may have utility as phylogenetic markers. In general, venom CRISP relationships reflect established phylogenetic relationships among the species from which they are derived. By analogy with the three-finger toxins of reptile venoms, which also have a highly conserved protein scaffold stabilized by disulfides, one can expect that venom CRISPs will also show myriad pharmacological activities. Future efforts should be directed toward the elucidation of these activities, as they are an excellent protein family for structure-activity studies.

I. INTRODUCTION

Cysteine-rich secretory proteins (CRISPs) are a widely distributed family of proteins that have been isolated from numerous animal tissues, including reptile venoms (e.g., Hill and Mackessy, 2000; Yamazaki and Morita, 2004; Fry et al., 2006), a cone snail venom (Milne et al., 2003), tissues of several other invertebrates (e.g., Schreiber et al., 1997; Ookuma et al., 2003), and a suite of nonreptilian vertebrate tissues. Nonreptilian vertebrate tissues represent the source from which CRISPs were originally discovered and described. The list of tissues containing CRISPs continues to grow, and now includes tissues such as the pancreas, the mammalian male reproductive tract, and the salivary glands (Kierszenbaum et al., 1981; Haendler et al., 1993; Kratzschmar et al., 1996;

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Schambony et al., 1998, 2003; Roberts et al., 2006). Although the function of many of the CRISPs is not known, some venom CRISPs have shown a diverse array of biological activities, including inhibition of several types of ion channels (Brown et al., 1999; Nobile et al., 1994, 1996; Yamazaki et al., 2002a; Wang et al., 2005), induction of hypothermia in prey animals (Mocha-Morales, 1990), and specific proteolysis (Milne et al., 2003). ~~However, many of the venom CRISPs also have no currently identifiable function.~~

CRISP proteins were originally described from the mammalian male reproductive tract (e.g., Kierszenbaum et al., 1981), but they have since been found in a number of vertebrate tissues such as neutrophils, plasma, salivary gland, pancreas, ovary, thymus, and colon (Kratzschmar et al., 1996, and references therein; Udby et al., 2002, and references therein). Several reviews (Kratzschmar et al., 1996; Udby et al., 2002; Jalkanen et al., 2005) provide summaries of the types and expression locations of nonreptilian CRISPs. In brief, CRISPs were historically placed into one of three primary types: CRISP-1, CRISP-2, and CRISP-3. CRISP-1, also known as sperm-coating glycoprotein DE or acidic epididymal glycoprotein (AEG), is expressed primarily in the epididymis and is thought to be involved in gamete fusion. CRISP-2, also known as *Tpx-1*, is produced in the testes and is likely involved in the interaction of spermatogenic and Sertoli cells. CRISP-3 is the most widely distributed of the three types and has been hypothesized to be involved in innate immune response. ~~Recently, a~~ fourth type of nonreptilian CRISP, known as CRISP-4, has been discovered in the mammalian epididymis (Jalkanen et al., 2005; Nolan et al., 2006).

Previously characterized venom CRISPs have shown a wide variety of functionalities, including inhibition of several types of ion channels (Brown et al., 1999; Nobile et al., 1994, 1996; Yamazaki et al., 2002a; Wang et al., 2005), induction of hypothermia in prey animals (Mocha-Morales, 1990), and specific proteolysis (Milne et al., 2003). However, for the majority of CRISPs isolated from reptile venoms, the function and biological role in venom are unknown.

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II. DISTRIBUTION OF CRISPs AMONG REPTILE VENOMS

Venom CRISPs have been isolated and characterized from the venoms of all three major venomous snake families (for a review see Yamazaki and Morita, 2004)—Colubridae (Hill and Mackessy, 2000; Mackessy, 2002; Mackessy et al., 2006; Yamazaki et al., 2002b; Fry et al., 2006), Elapidae (Brown et al., 1999, 2003; Osipov et al., 2001, 2005; Yamazaki et al., 2002a, 2002b, 2003; Jin et al., 2003; Wang et al., 2004a, 2004b, 2005; Fry et al., 2006), and Viperidae (Chang et al., 1997; Yamazaki et al., 2002b, 2003; Jin et al., 2003; Wang et al., 2004b; Guo et al., 2005; Shikamoto et al., 2005; Fry et al., 2006)—and from lizard venom and saliva (Mocha-Morales et al., 1990; Fry et al., 2006). Interestingly, some species appear to have a suite of different CRISP isoforms that are expressed simultaneously (Jin et al., 2003; Osipov et al., 2005; Fry et al., 2006). As mentioned in a review by Mackessy (2002), CRISPs are likely more widely distributed in the secretory products of venomous reptiles than has been previously recognized.

III. BIOLOGICAL ACTIVITY OF VENOM-DERIVED CRISPs

Venom CRISPs demonstrated a wide variety of biological effects, including blockage of potassium currents in neurons (Nobile et al., 1994), blockage of calcium currents in neurons (Nobile et al., 1996), binding to cyclic nucleotide-gated ion channels in both photoreceptor cells and olfactory neurons (Brown et al., 1999, 2003; Yamazaki et al., 2002a), blockage of BK_{Ca} channels (Wang et al., 2005), blockage of Ca^{2+} release from the sarcoplasmic reticulum (via ryanodine receptors) of both cardiac and skeletal muscle (Morrissette et al., 1995), blockage of vascular smooth muscle contraction (Yamazaki et al., 2002b), specific proteolysis (Milne et al., 2003—a cone snail venom toxin), induction of hypothermia in prey animals (Mocha-Morales et al., 1990), and lethality (Mocha-Morales et al., 1990). Despite the diversity of functionalities listed above, many venom

CRISPs currently have no identifiable function and apparently no acutely toxic effects (Chang et al., 1997; Yamazaki et al., 2002b; Jin et al., 2003; Osipov et al., 2005; Heyborne and Mackessy, unpublished data).

Several reptile venoms have contained multiple CRISP isoforms (Jin et al., 2003, Osipov et al., 2005; Fry et al., 2006). With this in mind, it would be interesting to examine the venom of *Heloderma horridum* more thoroughly. The CRISP from the venom of this species (helothermine) has very diverse functionalities, including the blockage of multiple types of ion channels (Nobile et al., 1994, 1996) and the induction of hypothermia in prey (Mocha-Morales et al., 1990). Given the diversity of biological activities reported for helothermine, one might hypothesize there to be more than a single CRISP isoform in the venom of this species, each with a slightly different sequence and thus biological activity.

IV. CRISP STRUCTURE

Despite the lack of functional data for many of the CRISPs, the structural chemistry of the venom CRISPs is quite well understood, following the recent crystallization of three such molecules (Guo et al., 2005; Shikamoto et al., 2005; Wang et al., 2005). These venom CRISP structures have shown this family of proteins to have a highly conserved primary, secondary, and even tertiary structure. Due to the high levels of structural conservatism, new members of this family are easily identifiable based on their primary structure alone.

A. Primary Structure

Cysteine-rich secretory proteins were first named because of the large number of cysteine residues found in the C-terminal portion (the cysteine-rich domain—see below). However, because many venom proteins contain numerous cysteines and disulfides, Kini et al. (2001) suggested the name *helveprin* (derived from *helothermine-like venom protein*) to distinguish venom CRISPs from other cysteine-rich venom proteins. Like the phospholipases A_2 (PLA_2s) and three-finger toxins (3FTxs) (see Chapters 5 and 10, this volume), venom CRISPs have a constrained structure defined by sixteen cysteines participating in eight highly conserved disulfide bonds (Table 16.1).

B. Crystallography

The first comprehensive structural analysis of a venom CRISP was conducted on the protein stecrisp from the venom of *Trimeresurus stejnegeri* (Guo et al., 2005). Crystallization of this molecule showed stecrisp to be comprised of two distinct regions connected by a folded hinge or bridge (Figure 16.1). The first of these regions, from the N-terminus of the molecule, was called the PR-1 domain due to its structural homology to the plant pathogenesis group 1 protein family. Known PR-1 crystal structures, including P14a described by Fernández et al. (1997), have shown a characteristic α/β/α sandwich element, which was also seen in stecrisp. The second region, from the C-terminal portion of stecrisp, was called the cysteine-rich domain (CRD) due to the high proportion of cysteine residues in this part of the molecule. Previous work on venom CRISPs had shown a strictly conserved set of sixteen cysteine residues throughout the molecule (Yamazaki and Morita, 2004). Guo et al. (2005) showed these sixteen residues form eight paired disulfide bonds in stecrisp. Three of these were found in the PR-1 domain, two in the hinge or bridge, and three in the cysteine-rich domain. Subsequent crystallization of two additional venom CRISPs (natrin from the venom of *Naja atra*, Wang et al., 2005, and triflin from *Trimeresurus flavoviridis*, Shikamoto et al., 2005) confirmed the presumed structural homology of venom CRISPs, as natrin and triflin also showed the two bridge-connected domains, as well as the α/β/α sandwich element in the PR-1 domain and the eight conserved disulfide bonds.

TABLE 16.1
Alignment (ClustalX v1.81) of 49 Cysteine-Rich Secretory Proteins from Venoms and Other Sources

O19010	_HORSE	-QDPGFAALSITKSEVQKEIVNKHNDLRRTVSPLASNMLKMQWDSKTATNAQNWANKCLLQ-HSKAEDRAVGTM-KCGENLFMSSIPNSWSDAIQNWHDEVHDFKYGVGPKTPN-AVVGHYTQVVWYSSYRVGCGIAYC----PKQ--GT	140
Q8HXA1	_HORSE	-QDPGFAALSITKSEVQKEIVNKHNDLRRTVSPLASNMLKMQWDSKTATNAQNWANKCLLQ-HSKAEDRAVGTM-KCGENLFMSSIPNSWSDAIQNWHDEVHDFKYGVGPKTPN-AVVGHYTQVVWYSSYRVGCGIAYC----PKQ--GT	140
Q5U8Z9	_HUMAN	-KDPAFTALLTTQLQVQREIVNKHNELRKAVSPPASNMLKMEWSREVTTNAQRWANKCTLQ-HSDPEDRKT-ST-RCGENLYMSSDPTSWSSAIQSWYDEILDFVYGVGPKSPN-AVVGHYTQLVWYSTYQVGCGIAYC----PNQ--DS	139
Q3MJ82	_HUMAN	DKDPAFTALLTTQTQVQREIVNKHNELRRAVSPPARNMLKMEWNKEAAANAQKWANQCNYR-HSNPKDR-MTSL-KCGENLYMSSAPSSWSQAIQSWFDEYNDFDFGVGPKTPN-AVVGHYTQVVWYSSYLVGCGNAYC----PNQK--V	140
Q2XXR1	_VARAC	--SLDLDDLMTTNPEIQNEIINKHNDLRRTVDPPAKNMLKMSWDNIIAESAKRAALRCNQNEHTPVSGRTIGGV-VCGENYFMSSNPRTWSFGIQSWFDERNYFKFGFGPTRAG-VMVGHYTQVVWYKSYKMGCAINLC----PNE---P	139
Q2XXR2	_VARAC	--SLDLDDLMTTNPEIQNEIINKHNDLRRTVDPPAKNTLKMSWDNTIAESAKRAALRCNQNEHTPVSGRTIGGV-VCGENYFMSSNLRTWSFGIQSWFDERNYFKFGFGPTRAG-VMVGHYTQVVWYKSYKMGCAINLC----PNE---P	139
Q2XXR0	_VARAC	--SLDLDDLMTTNPEIQNEIINKHNDLRRTVDPPAKNMLKMSWDNIIAESAKRAALRCNQNEHTPVSGRTIGGV-VCGENYFMSSNPRTWSFGIQSWFDERNYFKFGFGPTRAG-VMVGHYTQVVWYKSYKMGCAINLC----PNE---P	139
Q2XXP2	_VARVA	--SLDLDDLMTTNPEIQNEIINKHNDLRRTVDPPAKNMLKMSWDNIIAESAKRAALRCNYKEHTSIAERTIGGV-VCGENYFMSSNPRTWSSSIQSWFDERNNFMFGFGPTIPG-VMVGHYTQVVWYKSYKVGCAINLC----PAQ---S	139
Q91055	_HELHO	--SPKLPGLMTSNPDQQTEITDKHNNLRRIVEPTASNMLKMTWSNKIAQNAQRSANQCTL-EHTSKEERTIDGV-ECGENLFFSSAPYTWSYAIQNWFDERKYFRFNYGPTAQN-VMIGHYTQVVWYRSYELGCAIAYC----PDQP--T	139
Q2XXQ2	_ENHPO	--DVNFESESPRRTEKQTEIVDMHNSFRRSVNPTARNMLKMEWYPEAADNAERWAYQCIYD-HSANSERVIGGI-QCGENIYKSSNPRAWTEIIQSWYDEIQNFEYGVGANPPG-SVIGHYTQIVWYKSYRIGCAAAYC----PSY---P	138
Q2XXQ3	_ENHPO	--DVNFESESPRRTEKQTEIVDMHNSFRRSVNPTARNMLKMEWYPEAADNAERWAYQCIYD-HSANSERVIGGI-QCGENIYKSSNPRAWTEIIQSWYDEIQNFEYGVGANPPG-SVIGHYTQIVWYKSYRIGCAAAYC----PSY---P	138
Q2XXQ1	_LEIMD	--TVDFDSESPRRPEKQREIVNMHNSLRRSVSPTASNMLKMEWYPEAASNAERWAYNCITG-HSSNPSRVIDGI-QCGENIYMSPVPITWTEIIQIWYDENKNFVYGVGANPPG-SMIGHYTQIVWYKSYRIGCAAVYC----PST---Y	138
Q09GJ9	_PHIOL	--LVDFNSESPRRPEIQRVIVDTHNSYRRTVSPTASNMLRMEWYAEAAANAERWASLCAYD-HSQNSDRVLDGI-QCGENIYMSSNPRSWTEIMQSWYDEYKNFDFGYGANPPG-SVIGHYTQIVWYKSYRIGCAAYYC----PSS---L	138
Q2XXQ0	_LIOPO	--RIDIDSQSTRRPEIQNLIVDTHNSYRRSVSPTATNMLKMEWYPEAAANAERWAYQCVYD-HSQKYERVLDGI-QCGENIYMYSEFRPWTEVMQSWYDEYRNFIFGVGAHPAN-ALIGHYTQIVWYKSYRVGCAIANC----PSY---P	138
Q7ZT98	_OPHHA	--NVDFNSESTRRQKKQKEIVDLHNSLRRSVSPTASNMLKMQWYPEAASNAERWASNCNLG-HSPDYSRVLEGI-ECGENIYMSSNPRAWTEIIQLWHDEYKNFVYGVGANPPG-SVTGHYTQIVWYKTYRIGCAVNYC----PSS---E	138
Q7T1K6	_NAJAT	--NVDFNSESTRRKKKQKEIVDLHNSLRRRVSPTASNMLKMEWYPEAASNAERWANTCSLN-HSPDNLRVLEGI-QCGESIYMSSNARTWTEIIHLWHDEYKNFVYGVGANPPG-SVTGHYTQIVWYQTYRAGCAVSYC----PSS---A	138
Q2XXQ5	_DISTY	--NVDFNSESPRIKAKQREIVDKHNAFRRSVRPTASNMLRMEWYSEAASNAERWAYRCILD-HSPKTSRILNGI-KCGENIYMSSIPMTWIDIIKLWHDEYKNFIYGVGANPPG-SVIGHYTQIVWYKSYRVGCAASYC----PSS---S	138
Q2XXQ6	_DISTY	--NVDFNSESPRIKAKQREIVDKHNAFRRSVRPTASNMLRMEWYSEAASNAERWAYRCILD-HSPKTSRILNGI-KCGENIYMSSIPMTWIDIIKLWHDEYKNFIYGVGANPPG-SVIGHYTQIVWYKSYRVGCAASYC----PPS---S	138
Q2XXQ4	_DISTY	--NVDFNSESPRIKAKQREIVDKHNAFRRSVRPTASNMLRMEWYSEAASNAERWAYRCVLD-HSPETSRILNGI-QCGENIYMSSIPRTWIDIIKLWHDEYKNFIYGVGANPPG-SIIGHYTQIVWYKSYRIGCAASYC----PSS---S	138
Q2XXP5	_TELDH	--NVDFNSESPRRKNKQKEIVDMHNSLRRSVKPTASNMLRMEWYSEAASNAERWAYRCILN-HSPESSRILGGI-KCGENIYMSSNPRKWTEVIQLWYDEYKNFVYGIGANPRG-SVIGHYTQIVWYKSYRIGCAAYYC----PSS---S	138
Q2XXP4	_TRIBI	--NVDFNSESPRRPEKQKEIVDRHNSFRRSVRPTASNMLKMEWYSEAASNAERWAYRCNLG-HSPDSSRILDGI-KCGENIYMSSNPRAWTEILQLWYDEYKNFVYGVGANPPG-SVTGHFSQMVWYKSYRIGCAAAYC----PSS---G	138
Q8JGT9	_RHATT	--NVDFNSESPRNPGKQQEIVNIHNSFRRSVRPTARNMLKMEWYSEAASNAERWAYQCAYD-HSPRSSRILNGI-QCGENIFMASDPWAWTSIIQDWYDEYRNFVYGVGANPPD-SVTGHYTQIVWYKSYLVGCAAAYC----PSS---L	138
P79845	_TRIMU	--NVDFDSESPRKPEIQNEIIDLHNSLRRSVNPTASNMLKMEWYPEAAANAERWAYRCIES-HSSRDSRVIGGI-KCGENIYMSPYPAKWTDIIHAWHGEYKDFKYGVGAVPSN-AATGHYTQIVWYKSYRGGCAAAYC----PSS---K	138
Q8JI39	_TRIFL	--NVDFDSESPRKPEIQNEIIDLHNSLRRSVNPTASNMLKMEWYPEAAANAERWAYRCIES-HSSRDSRVIGGI-KCGENIYMATYPAKWTDIIHAWHGEYKDFKYGVGAVPSD-AVIGHYTQIVWYKSYRAGCAAAYC----PSS---K	138
Q7ZZN9	_TRIJE	--NVDFDSESPRKPEIQNEIIDLHNSLRRSVNPTASNMLKMEWYPEAAANAERWAYGCIES-HSSRDSRVIEGI-KCGENIYMSPYPMKWTDIIHAWHGEYKDFKYGVGAVPSD-AVVGHYTQIVWYKSYRIGCAAAYC----PSA---E	138
P60623	_TRIST	--NVDFDSESPRKPEIQNEIVDLHNSLRRSVNPTASNMLRMEWYPEAADNAERWAYRCIES-HSSYESRVIEGI-KCGENIYMSPYPMKWTDIIHAWHDEYKDFKYGVGADPPN-AVTGHYTQIVWYKSYRIGCAAAYC----PSS---P	138
Q8JI40	_AGKHA	--NVDFDSESPRKPEIQNEIVDLHNSLRRSVNPTASNMLKMEWYPEAAANAERWAYRCIED-HSSPDSRVLEGI-KCGENIYMSPIPMKWTDIIHIWHDEYKNFKYGIGADPPN-AVSGHFTQIVWYKSYRAGCAAAYC----PSS---E	138
Q7ZT99	_CROAT	--SVDFDSESPRKPEIQNKIVDLHNFLRRSVNPTASNMLKMEWYPEAAANAERWAYRCIES-HSPRDSRVLGGI-KCGENIYMSPVPIKWTEIIHAWHGENKNFKYGIGAVPPN-AVTGHFSQVVWYKSYRIGCAAAYC----PSS---K	138
Q7ZTA0	_AGKPI	--SVDFDSESPRKPEIQNQIVDLHNSLRRSVNPTASNMLKMEWYPEAAANAERWAYRCIES-HSPRNSRVLGGI-KCGENIYMSSIPIKWTEIIHAWHGENKNFKYGIGADPPN-AVIGHFTQIVWYKSYLVGCAAAYC----PSS---E	138
B0VXV6	_SISCA	--SVDFDSESPRKPEIQNKIVDLHNSLRRSVNPTASNMLKMEWYSEAAANAERWAYRCIES-HSPRDSRVLEGI-KCGENIYMSSVPMKWTEIIHIWHGENKNFKYGIGADPPN-AVTGHYTQIVWYKSYRAGCAAAYC----PSL---E	138
A7X4T8	_CAURH	--ANPSY-AVVGHYTQIVWYKSDRIGCAAAYC----PSS---V	33
Q7ZZN8	_NAJAT	--TVDFASESSNKRENQKQIVDKHNALRRSVRPTARNMLQMEWNSNAAQNAKRWADRCSFA-HSPPHLRTVGKI-GCGENLFMSSQPYAWSRVIQSWYDENKKFVYGVGANPPG-SVIGHYTQIVWYNSHLLGCGAAKC-----SS----	136
Q8JI38	_LATSE	--TVDFASESSNKRENQKEIVDKHNALRRSVRPTARNMLQMEWNSDAAQNAQRWADRCSFA-HSPSHLRTVGKF-SCGENLFMSSQPYAWSRVIQSWYDENKNFIYDVGANPPG-SVIGHYTQIVWYKSHLLGCAAARC-----SS----	136
Q8AVA3	_PSEPO	--TVDFASESSNKKNYQKEIVDKHNALRRSVKPTARNMLQMKWNSHAAQNAKRWADRCTFA-HSPPNTRTVGKL-RCGENIFMSSQPFPWSGVVQAWYDEIKNFVYGIGAKPPG-SVIGHYTQVVWYKSHLIGCASAKC-----SS----	136
Q8AVA4	_PSEAU	--TADFASESSNKKNYQKEIVDKHNALRRSVKPTARNMLQMKWNSRAAQNAKRWANRCTFA-HSPPNKRTVGKL-RCGENIFMSSQPFPWSGVVQAWYDEIKNFVYGIGAKPPG-SVIGHYTQVVWYKSYLIGCASAKC-----SS----	136
Q8UW11	_LAPHA	--TVDFASESSNKKDYQREIVDKHNALRRSVKPTARNMLQMKWNSHAAQNAKRSADRCTFA-HSPEHTRTVGKF-RCGENIFMSSQPFAWSGVVQAWYDEIKNFVYGIGAKPPG-SVIGHYTQIVWYKSHLLGCASAKC-----SS----	136
Q8UW25	_LAPHA	--TVDFASESSNKKDYRREIVDKHNALRRSVKPTARNMLQMKWNSRAAQNAKRSADRCTFA-HSPEHTRTVGKF-RCGENIFMSSQPFAWSGVVQDWYDEIKNVVDGIGAKPPG-SVIGHYTQIVWYKSHLLGCASAKC-----SS----	136
Q3SB04	_9SAUR	--TVDFASESSNKKDYQKEIVDKHNALRRSVKPTARNMLRMEWNSHAAQNAKRWADRCTFA-HSPPHTRTVGKL-RCGENIFMSSQPFAWSGVVQAWYDEVKKFVYGIGAKPPG-SVIGHYTQVVWYKSHLLGCASAKC-----SS----	136
Q3SB03	_HOPST	--TVDFASESSNKKDYQKEIVDKHNALRRSVKPTARNMLRMKWNSRAAQNAKRWADRCTFA-HSPPHTRTVGKL-RCGENIFMSSQPFAWSGVVQAWYDEVKKFVYGIGAKPPG-SVIGHYTQVVWYKSHLLGCASAKC-----SS----	136
A8S6B6	_AUSSU	--TVDFASESSNKKDYRKEIVDKHNALRRSVKPTARNMLRMEWNSRAAQNAKRWADRCTFA-HSPPHTRTVGKL-RCGENIFMSTQPFAWSGVVQAWYDEVKKFVYGIGAKPPG-SVIGHYTQVVWYKSHLLGCASAKC-----SS----	136
Q2XXP9	_OXYMI	--TVDFASESSNKKDYRKEIVDKHNDLRRSVKPTARNMLQMKWNSRAAQNAKRWANRCTFA-HSPPYTRTVGKL-RCGENIFMSSQPFAWSGVVQAWYDEVKKFVYGIGAKPPS-SVIGHYTQVVWYKSHLLGCASAKC-----SS----	136
Q3SB07	_9SAUR	--TVDFASESSNKKDYRKEIVDKHNDLRRSVKPTARNMLQMKWNSRAAQNAKRWANRCTFA-HSPPYTRTVGKL-RCGENIFMSSQPFAWSGVVQAWYDEVKKFVYGIGAKPPS-SVIGHYTQVVWYKSHLLGCASAKC-----SS----	136
Q3SB06	_OXYMI	--TVDFASESSNKKDYRKEIVDKHNDLRRSVKPTARNMLQMKWNSRAAQNAKRWANRCTFA-HSPPYTRTVGKL-RCGENIFMSSQPFAWSGVVQAWYDEVKKFVYGIGAKPPS-SVIGHYTQVVWYKSHLLGCASAKC-----SS----	136
Q3SB05	_PSETE	--TVDFASESSNKNDYQKEIVDKHNDLRRSVKPTARNMLQMKWNSRAAQNAKRWANRCTFA-HSPPYTRTVGKL-RCGENIFMSSQPFAWSGVVQAWYDEVKKFVYGIGAKPPS-SVTGHYTQVVWYKSHLLGCASAKC-----SS----	136
A6MFK9	_DEMVE	--TVDFASESSNKGENQKQIVKKHNALRRSVKPPARNMLQMEWNSRAAQNAKRWAERCSFT-HSPPSLRTVGKL-RCGENLLQSSQPLPWSKVVQAWYDENKNFVYGIGAKPPG-SVVGHYTQVVWYKSRLLGCASVKC-----SP----	136
Q642T6	_XENTR	--DPPFSSISTDNVTVTQIIINAHNNYRRNASPSARNMLKMVWNKDAAINAASWAATCSES-HSPSDKRTIPGF-GCGENLYMASYPASWEEAVKGWYSEYNDFQYGVGPKSPG-LVTGHYTQVMWYNSYMVGCSVSYC----PKS---P	138
Q801Z0	_XENLA	---GISLSLSTYISTVRQTIVDTHNSYRRNANPTAQNMLKMMWNEDTAQNAGNWSATCSFT-HSTSNQRTIPGF-NCGENIYMATYPASWEEAIQAWYSEGEFFNMEWDPLLPGKDPQGHYTQLMWYNSYMVGCAVSYC----PAN---Y	138
Q5BL94	_XENTR	MKNVPFSALSTDLESNRQSILNVHNELRRNANPPPSNMLKMVWSDLAAKSAAKWANSCKQY-HSLKPERTIPGF-SCGENLFMASYKASWEDVIRAFYSEIEDFLYGKGAKEVG-LQILHFTQVMWFSSWLVGCAAAQC----PITDH-S	142
B0WCQ0	_CULQU	--KLPTKVISPRTRKVQRRIVVLHDFFRTKVVPPAANMLSMKWHHGAARSAQKWADQCRLLTHDSPKGRWIDNYGACGQNIFVSTHKVPWLFALRTWFLERQNFTYA-SPKNDL-VAVGHYTQMVWAATHKVGCGLAKCARGGPRG---K	143
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TABLE 16.1 (continued)
Alignment (ClustalX v1.81) of 49 Cysteine-Rich Secretory Proteins from Venoms and Other Sources

Note: Conserved (100%) cysteine residues are indicated by an arrow. The PR-1 domain, hinge region, and cysteine-rich domain (based on stecrisp; Guo et al., 2005) are also indicated. Individual CRISPs are listed by their GenBank accession numbers; see appendix for species names.

C. Structure-Function Relationships

The broad distribution and variable functionality of CRISP proteins found in reptile venoms has been equated with those of PLA_2s (Mackessy 2002), which also have a broad range of functions and taxonomic distribution. Kini (2003) suggested that the range in functionalities seen among the PLA_2s may be due to separate pharmacological sites and active sites on each of the molecules. The pharmacological site is thought to be responsible for cell- or molecule-specific binding in the target tissue, while the active site is responsible for actual catalytic activity. Perhaps a similar model would be applicable to the venom CRISPs. Based on x-ray crystallography with a venom CRISP, Guo et al. (2005) commented on the possibility of "functional separation" between two main domains of the molecule, thus strengthening the argument for a similarity in diverse functionality on a conserved structural scaffold, as seen in the PLA_2s and 3FTxs.

The functional sites of venom CRISPs have not been absolutely determined for any molecule. The wide variety of functionalities, as discussed above, would suggest that there may be multiple functional sites or residues. Yamazaki et al. (2002a) described the CRISP toxins pseudechetoxin and pseudecin (both from Australian elapids) that were found to block cyclic nucleotide-gated ion channels. Based on differences in blocking affinity between these two molecules, the researchers contended that a string of basic residues (Lys167, Lys174, Arg175), which were

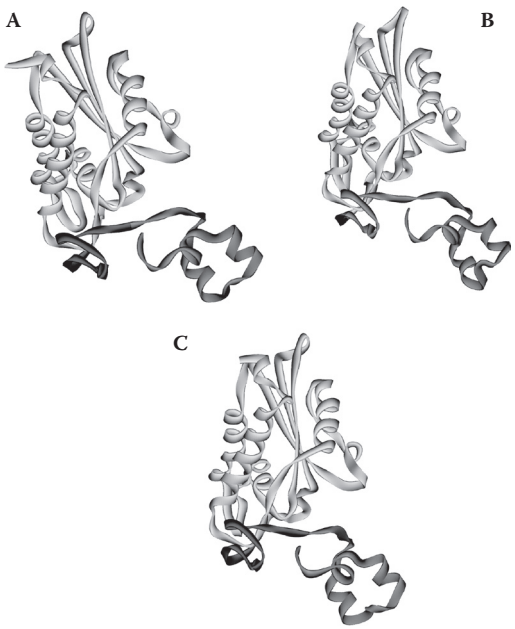

FIGURE 16.1 ***(A color version of this figure follows page XXX.)*** Snake venom CRISP structures generated by SWISS-MODEL. (A) Stecrisp (GenBank/EBI accession number 1RC9A). (B) Triflin (1WVRA). (C) Natrin (1XTAA). Ribbon color is used to differentiate structural domains as described by Guo et al. (2005). ~~Yellow,~~ N-terminal PR-1 domain; ~~red,~~ bridge region; ~~blue,~~ C-terminal cysteine-rich domain. Note the high degree of structural homology in all three molecules.

found in pseudechetoxin but were lacking (except for Arg166) in pseudecin, may interact electrostatically with the CNG channels, leading to the observed blockage. Yamazaki et al. (2003) proposed that the region near and including residues 184–189 may be the functional region in snake venom CRISPs found to block smooth muscle contraction (ablomin, triflin, latisemin, piscivorin, and catrin). Based on differences in the binding affinity of these venom CRISPs, residues Phe189 and Glu186 were considered likely candidates for the observed channel blocking activity. Wang et al. (2005) and Guo et al. (2005) both conducted crystallization studies of snake venom CRISPs (natrin and stecrisp, respectively) and noted that previously suggested functional residues (Lys175, Arg176, Glu186, and Phe189—see above) are all part of "exposed solvent loop I" (Wang et al., 2005) ("N-terminal loop"; Guo et al., 2005) in the cysteine-rich domain of the molecules. They suggested that this loop may prove to be the region of interface between CRISPs and other molecules due to the high levels of variability found here (which would help explain the diverse functionalities observed) and its presumed ease of interaction with other molecules because of its exposed conformation. In studies of the only known venom CRISP protease, Milne et al. (2003) suggested that residues Ser80, Glu115, and His130 may be part of a catalytic triad (analogous to those of known serine proteases) due to their proximity and location within an electronegative cleft. Catalytic triads of this type not only act enzymatically, but also likely charge-stabilize the intermediate stages of the molecular substrate (Carter and Wells, 1988).

V. USES IN PHYLOGENETIC HYPOTHESIS TESTING

Due to the wide distribution of the CRISP family among reptilian oral secretions, including some lizard species that have not previously been considered venomous (Fry et al., 2006), this protein family may be especially useful in the study of venom evolution. Other researchers (Yamazaki

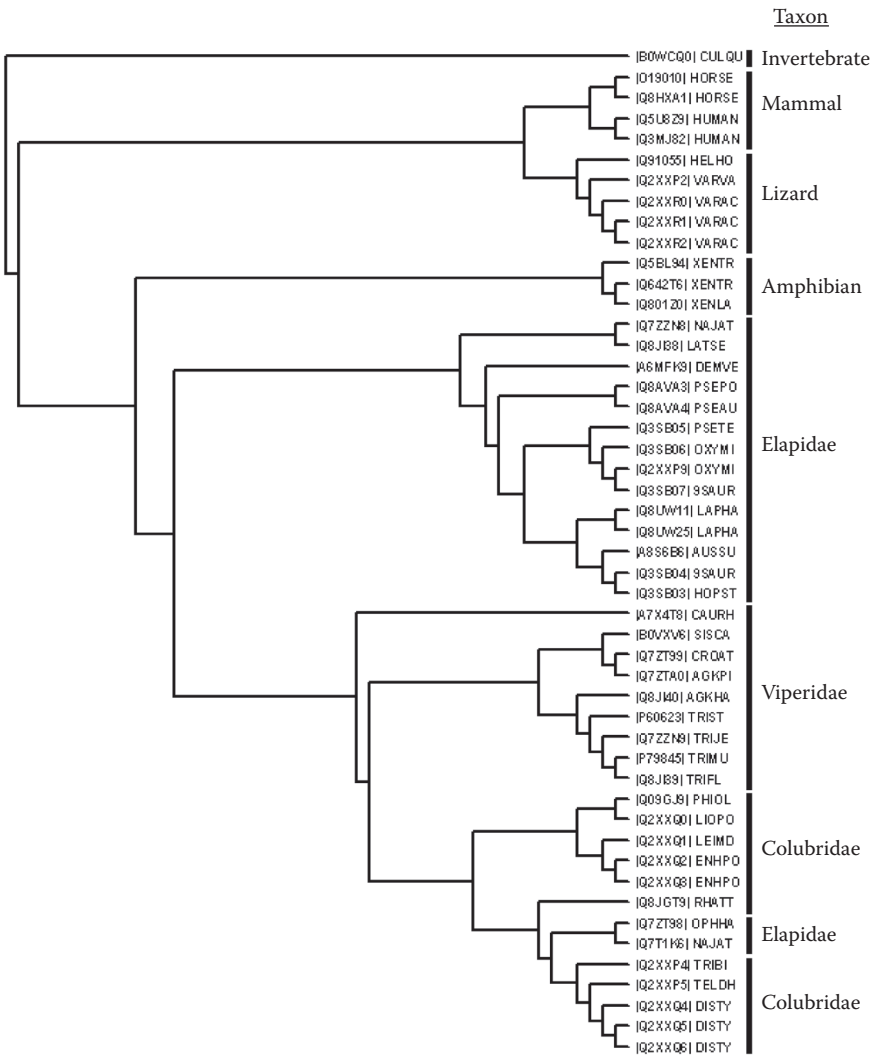

FIGURE 16.2 Phylogram of sequence similarity relationships between venom and other CRISPs. The neighbor-joining tree was drawn using ClustalX v1.81 alignment and TreeView 1.6.6. Note that toxin clades follow generally accepted taxonomic groups, with the exception of two elapid taxa, which cluster with the Colubridae.

et al., 2003; Fry and Wüster, 2004) have recognized the potential use of CRISPs and have used CRISP sequence data in phylogenetic analyses. However, the number of CRISP sequences now available has grown significantly. A BLAST search of available sequences (2007) revealed forty-nine CRISP sequences, most of which are derived from reptile venom gland DNA sequences (see Table 16.1 and appendix). These forty-nine sequences were aligned and a neighbor-joining tree was drawn using ClustalX 1.81 and TreeView 1.6.6. (Figure 16.2). In general, sequence similarities follow phylogenetic affinities, with an exception that two elapid taxa (*Ophiophagus* and *Naja atra*) cluster within the Colubridae.

There are two additional reports of CRISP antibody screening of crude venoms. The first of these reports (Yamazaki et al., 2002b) gives few details regarding methodology or diversity of species screened. However, the authors do note the detection of CRISP-like proteins in the venoms of *Agkistrodon blomhoffi*, *Trimeresurus flavoviridis*, and *Laticauda semifasciata* when screened (via

Western blotting or ELISA) with anti-tigrin antibodies. The second study, conducted by the same group (Yamazaki et al., 2003), documents much wider screening (via ELISA) of snake venoms using anti-triflin antibodies. A total of fifteen species were screened, with all but two (*Notechis scutatus* and *Oxyuranus scutellatus*) showing positive reactivity. Of note is the fact that a second *Notechis* species, *N. ater*, was screened as part of the current study and also showed no reactivity toward anti-triflin, but did show positive activity toward anti-tigrin antibodies. Finally, because these fifteen species were screened using ELISA, the results should be interpreted with caution due to the high levels of apparent nonspecific interactions that we have observed.

Yamazaki et al. (2003) also performed a phylogenetic analysis of CRISP sequences known at that time. As this analysis was performed a number of years ago, the data set they utilized was much smaller than the data set presented here. They also utilized a different method (unweighted pair group method) to construct a phylogenetic tree, but their results were similar to those reported here. They reported the identification of a monophyletic viperid clade and a monophyletic elapid clade. One notable exception was the genus *Ophiophagus*, which grouped with the viperids instead of the elapids. Because they only used a single colubrid sequence, from *Rhabdophis tigrinus tigrinus*, it was not possible to make conclusions regarding the taxonomic status of this group.

VI. CONCLUSIONS

There has been some confusion regarding the naming of CRISP venom proteins. The name cysteine-rich secretory protein is not specific enough to identify unambiguously members of this group, as many other families of secreted proteins are also cysteine-rich, such as the three-finger toxins and phospholipases A_2. Some authors (Chang et al., 1997; Jin et al., 2003) have proposed calling venom CRISPs cysteine-rich venom proteins (CRVPs), but this name is no less ambiguous for the same reasons stated above. Kini et al. (2001) advocated the use of the term *helveprins* as an alternative to either of the above. While this name is more descriptive, as it refers to the first venom CRISP isolated (helothermine) and indicates the source (venom), it has not been adopted generally.

Ohno et al. (1998) commented on the "functional prodigality associated with a structural economy" often seen among well-characterized snake venom toxins such as PLA_2s and 3FTxs. With increasing frequency, researchers have noted that venom toxins with very different biological functions often have a remarkably similar protein fold. Evolutionarily, the modification of a stable and biologically successful molecular scaffold for additional or novel functionalities, perhaps via a cassette-like exchange mechanism of specific toxin segments (ASSET; ~~Pahari~~ Fry et al., 2008) rather than the evolution of molecules *de novo*, is a more parsimonious mechanism for toxin diversification. It is apparent that CRISP venom proteins will similarly adhere to this model of structural conservatism. Undoubtedly, future studies of CRISP molecules will help to provide answers to structural and functional questions regarding this family of diverse proteins.

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APPENDIX: SPECIES SOURCES OF CRISPs USED IN ALIGNMENTS AND PHYLOGRAM

GenBank accession number, trivial name, and source species are provided for each CRISP.

Invertebrates

B0WCQ0	Catrin	*Culex quinquefasciatus* (southern house mosquito)

Vertebrates

Amphibians

Q642T6	Crisp2 protein	*Xenopus tropicalis* (western clawed frog)
Q5BL94	MGC108118 protein	*Xenopus tropicalis* (western clawed frog)
Q801Z0	Cysteine-rich secretory protein	*Xenopus laevis* (African clawed frog)

Reptiles

Lizards

Q91055	Helothermine precursor (HLTx)	*Heloderma horridum horridum* (Mexican beaded lizard)
Q2XXP2	CRISP-VAR10 (fragment)	*Varanus varius* (lace monitor)
Q2XXR2	CRISP-VAR3 (fragment)	*Varanus acanthurus* (ridge-tailed monitor)
Q2XXR1	CRISP-VAR4 (fragment)	*Varanus acanthurus* (ridge-tailed monitor)
Q2XXR0	CRISP-VAR5 (fragment)	*Varanus acanthurus* (ridge-tailed monitor)

Snakes

Colubridae

Q8JGT9	Tigrin precursor	*Rhabdophis tigrinus tigrinus* (tiger keelback snake)
Q2XXP4	CRISP-TRI1 (fragment)	*Trimorphodon biscutatus* (lyre snake)
Q2XXQ6	CRISP-DIS1	*Dispholidus typus* (boomslang)
Q2XXQ5	CRISP-DIS2	*Dispholidus typus* (boomslang)
Q2XXQ4	CRISP-DIS3	*Dispholidus typus* (boomslang)
Q09GJ9	Cysteine-rich secretory protein precursor (CRISP-PHI1) (CRISP-PHI2)	*Philodryas olfersii* (green snake)
Q2XXQ3	CRISP-ENH1	*Enhydris polylepis* (Macleay's water snake)
Q2XXQ2	CRISP-ENH2	*Enhydris polylepis* (Macleay's water snake)
Q2XXP5	CRISP-TEL1 (fragment)	*Telescopus dhara* (Egyptian catsnake)
Q2XXQ1	CRISP-LEI1 (fragment)	*Leioheterodon madagascariensis* (Malagasy giant hognose snake)
Q2XXQ0	CRISP-LIO1 (fragment)	*Liophis poecilogyrus* (water snake)

Viperidae

A7X4T8	CRISP-Cau1 (fragment)	*Causus rhombeatus* (rhombic night adder)
Q8JI40	Ablomin precursor	*Agkistrodon halys blomhoffi* (mamushi) (*Gloydius blomhoffii*)
Q8JI39	Triflin precursor	*Trimeresurus flavoviridis* (Habu) (*Protobothrops flavoviridis*)
P60623	Cysteine-rich secretory protein precursor (Stecrisp)	*Trimeresurus stejnegeri* (Chinese green tree viper)
P79845	Cysteine-rich venom protein precursor (TM-CRVP)	*Trimeresurus* (*Protobothrops*) *mucrosquamatus* (Taiwan habu)
Q7ZZN9	Cysteine-rich venom protein precursor (TJ-CRVP)	*Trimeresurus* (*Protobothrops*) *jerdonii* (Jerdon's pit-viper)
Q7ZT99	Catrin-1/2 precursor	*Crotalus atrox* (western diamondback rattlesnake)
B0VXV6	Cysteine-rich secretory protein isoform 2	*Sistrurus catenatus edwardsii* (desert massasauga)
Q7ZTA0	Piscivorin precursor	*Agkistrodon piscivorus piscivorus* (eastern cottonmouth)

Elapidae

Q7ZT98	Ophanin precursor (Opharin)	*Ophiophagus hannah* (king cobra)
Q7T1K6	Natrin-1 precursor (cysteine-rich venom protein 1) (NA-CRVP1) (protein G2a)	*Naja atra* (Chinese cobra)
Q8JI38	Latisemin precursor	*Laticauda semifasciata* (broad-banded blue sea snake) (Erabu sea snake)
Q3SB03	Pseudechetoxin-like protein precursor	*Hoplocephalus stephensii* (Stephens' banded snake)
Q8UW11	Cysteine-rich venom protein 2 precursor (CRVP)	*Lapemis hardwickii* (Hardwick's sea snake)
Q8UW25	Cysteine-rich venom protein 1 precursor (CRVP)	*Lapemis hardwickii* (Hardwick's sea snake)
Q3SB04	Pseudechetoxin-like protein precursor	*Notechis scutatus*
Q3SB05	Pseudechetoxin-like protein precursor	*Pseudonaja textilis* (eastern brown snake)
Q8AVA4	Pseudechetoxin precursor (PsTx)	*Pseudechis australis* (mulga snake) (king brown snake)
Q8AVA3	Pseudecin precursor	*Pseudechis porphyriacus* (red-bellied black snake)
Q3SB07	Pseudechetoxin-like protein precursor	*Oxyuranus scutellatus*
Q2XXP9	CRISP-OXY1	*Oxyuranus microlepidotus* (~~Inland~~ taipan)
Q3SB06	Pseudechetoxin-like protein precursor	*Oxyuranus microlepidotus* (inland taipan)
A8S6B6	CRISP precursor	*Austrelaps superbus* (Australian copperhead)
Q7ZZN8	Natrin-2 precursor (cysteine-rich venom protein 2) (NA-CRVP2) (protein G2b)	*Naja atra* (Chinese cobra)
A6MFK9	Cysteine-rich secretory protein precursor	*Demansia vestigiata* (lesser black whip snake)

Mammals

Q8HXA1	Cysteine-rich secretory protein 3	*Equus caballus* (horse)
O19010	Cysteine-rich secretory protein 3 precursor (CRISP-3)	*Equus caballus* (horse)
Q3MJ82	Cysteine-rich secretory protein 3	*Homo sapiens* (human)
Q5U8Z9	Testis-specific protein TPX1 e isoform (cysteine-rich secretory protein 2, isoform CRA_a) (CRISP2 protein) (testis-specific protein TPX1 d isoform) (testis-specific protein TPX1 b isoform)	*Homo sapiens* (human)