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Report

Identical Skin Toxins by Convergent Molecular Adaptation in Frogs

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Summary

The Tree of Life is rife with adaptive convergences at all scales and biological levels of complexity. However, natural selection is not likely to result in the independent evolution of identical gene products. Here we report such a striking example of evolutionary convergence in the toxic skin secretions of two distantly related frog lineages. Caeruleins are important decapeptides in pharmacological and clinical research [1] and are commonly believed to represent a single evolutionary class of peptides [2–4]. Instead, our phylogenetic analyses combining transcriptome and genome data reveal that independently evolved precursor genes encode identical caeruleins in *Xenopus* and *Litoria* frogs. The former arose by duplication from the *cholecystokinin* (*cck*) gene, whereas the latter was derived from the *gastrin* gene. These hormone genes that are involved in many physiological processes diverged early in vertebrate evolution, after a segmental duplication during the Cambrian period. Besides implicating convergent mutations of the peptide-encoding sequence, recurrent caerulein origins entail parallel shifts of expression from the gut-brain axis to skin secretory glands. These results highlight extreme structural convergence in anciently diverged genes as an evolutionary mechanism through which recurrent adaptation is attained across large phylogenetic distances.

Results

The biological effect of caerulein (pEQDY(SO_3)TGWMDF-NH_2) relies on its structural similarity to the C-terminal bioactive sites of cholecystokinin (CCK) and gastrin. As a potent ligand of CCK receptors [5], caerulein interferes with the physiological pathways of endogenous CCK and gastrin and causes acute pancreatitis, vomiting, diarrhea, decreased blood pressure, and inhibition of exploratory and feeding behavior [1]. After its discovery in skin secretions of the Australian frog *Litoria caerulea* [6], caerulein and isoforms (mutually referred to as "caeruleins") were found in a broad taxonomic range of other frogs, including the model species *Xenopus laevis* (Pipidae) [7–10]. Previous DNA cloning studies have identified three closely related, heavily exon-duplicated *caerulein* genes in *X. laevis* [11, 12]. We searched for novel *caerulein* genes and homologs via the skin transcriptomes of two additional frog species. *Litoria splendida* is a close relative of *L. caerulea* and is also known to secrete caerulein [1]. *Silurana tropicalis* (Pipidae) is a relative of *X. laevis*, and, although caeruleins have not been reported for this species, a previous study identified antimicrobial peptides with similarity to *Xenopus* caerulein precursors, suggesting an evolutionary link [13].

Novel Peptide Precursors in *S. tropicalis* and *L. splendida*

A cDNA library prepared from *S. tropicalis* skin contained transcripts encoding a novel protein of 91 amino acids. The central region of this protein bears ~89% sequence similarity to XT-6, a known *S. tropicalis* peptide with antimicrobial and hemolytic activities [13]. Screening of nano-liquid chromatography-tandem mass spectrometry spectra prepared from an *S. tropicalis* skin extract confirmed the excision and posttranslational modification of an XT-6 isoform (NLLGSLLKTGLKVGSNLL-NH_2; molecular weight = 1838.14) from this region, indicating that the protein represents an XT-6-like precursor (henceforth called XT6LP). Comparative alignment identified XT6LP as a structural intermediate between amphibian CCK and *X. laevis* caerulein precursors (represented in Figure 1 by caerulein 3 precursor, henceforth called XLCAE3P), in terms of both sequence similarity and exon number (Figures 1A and 1B). BLAST screening of the *S. tropicalis* genome (http://genome.jgi-psf.org/Xentr4/Xentr4.home.html) revealed that the *xt6lp* gene is composed of four exons and that the XT-6 isoform is encoded by flanking regions on exons 2 and 3. In addition, a single C-terminal region of XT6LP (encoded by exon 4) is highly similar to the bioactive site of CCK and the three caerulein-encoding regions of XLCAE3P.

Similar cDNA screening of the skin of *L. splendida* frogs yielded transcripts encoding a novel caerulein precursor of 67 amino acids (henceforth called LSCAE1P). This precursor shows surprisingly limited similarity to those of the pipid caeruleins, and most identical residues are restricted to the C-terminal caerulein-encoding region. Instead, BLAST searches identified the gastrin precursor of *Rana catesbeiana* (Ranidae) as most similar. Comparative alignment suggests that LSCAE1P represents a truncated homolog of amphibian gastrin (Figure 1C).

Parallel Origins of Caerulein Evolution

The distinct structures of *Xenopus* and *Litoria* caerulein precursors and their respective similarity to CCK and gastrin suggest their independent origins and, hence, convergent evolution of identical toxins. To test this scenario in a phylogenetic framework, we assembled a data set by aligning the precursor genes with *cck* and *gastrin* sequences of various vertebrates (data set 1). Maximum parsimony and maximum likelihood bootstrap analyses of data set 1 confirm that *cck* and *gastrin* diverged early in vertebrates [14] (Figure 2). The duplication event that gave rise to both hormones (node D1 in Figure 2) is recovered after the divergence of lampreys, defining the CCK/gastrin pair as a synapomorphy of jawed vertebrates. Despite low statistical support for several deep

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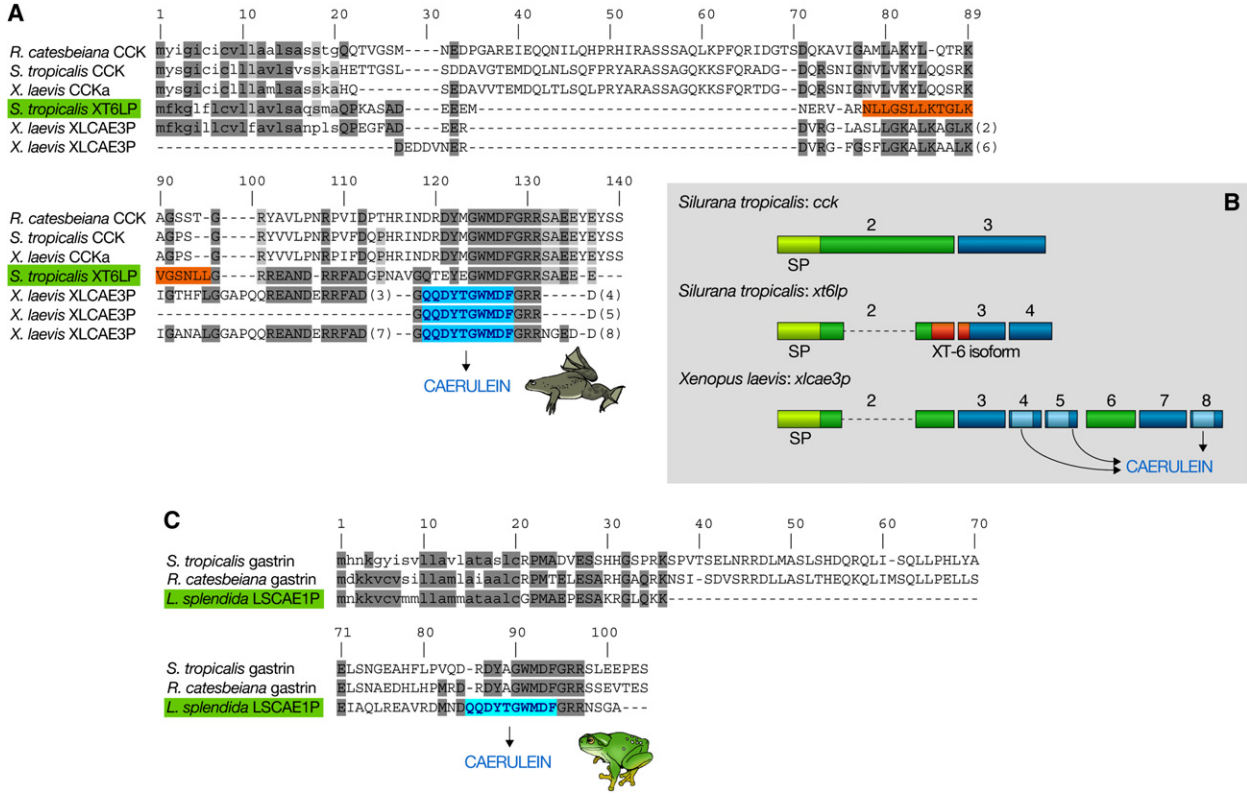

Figure 1. Comparative Alignments of the Newly Identified Precursors with Known Amphibian CCK, Gastrin, and Caerulein Precursors

(A) Amino acid sequence alignment of the *S. tropicalis* XT-6 like precursor (XT6LP) with amphibian CCK precursors and an *X. laevis* caerulein precursor (XLCAE3P). The sequence of XLCAE3P occupies multiple lines because duplicated exons were aligned to each other. Numbers in parentheses indicate XLCAE3LP exon ends.

(B) Gene structure comparison of the *xt6lp* gene with the *cck* and *xlcae3p* genes.

(C) Amino acid sequence alignment of the *Litoria splendida* caerulein 1 precursor (LSCAE1P) with amphibian gastrin.

The newly identified precursors are labeled in lime green. In (A) and (C), amino acid residues are highlighted as follows: dark gray, shared between caerulein precursors and others; light gray, shared between XT6LP and CCK precursors; orange, XT-6 isoform; blue, caerulein peptides. Predicted signal peptides are printed in lowercase. In (B), homologous exons and peptide regions are shown in the same color scheme. SP indicates signal peptide.

nodes (Figure 2), our analyses provide strong evidence for parallel origins of *caerulein* genes within the amphibian *cck* and *gastrin* clades. *xt6lp* is closely related to *xlcae3p* and their ancestral gene duplicated from *cck* within the pipid lineage. In addition, the close relationship of *lscae1p* with *R. catesbeiana gastrin* is corroborated, suggesting that this *caerulein* gene evolved from *gastrin* in a neobatrachian ancestor. Phylogenetic analysis of caeruleins has long been complicated by their short length and the absence of known neobatrachian precursors. The most parsimonious explanation for their presence in multiple distantly related frog families [7–10] was a single early origin in frogs. Accordingly, caeruleins have been considered to constitute a distant "evolutionary side branch" in the CCK/gastrin family [2–4]. To scrutinize our findings in a statistical framework, we evaluated alternative hypotheses of a single caerulein origin with likelihood-based phylogenetic tests [15]. Regardless of whether *caerulein* genes were assumed to represent a single branch within the *cck* clade, within the *gastrin* clade, or outside either hormone clade, our tests favor rejection of a single caerulein origin ($p < 0.001$ in all cases).

An alternative explanation for localized sequence similarity between distantly related genes is nonallelic recombination, when part of one gene is "copied" to the other through gene conversion. This is improbable in the case of caeruleins: parallel origins of their precursor genes as supported by our analyses imply that they never coexisted in the genome of a single ancestral species. Even if they had coexisted, their recombination would have been unlikely. Because *cck* and *gastrin* are located on different chromosomes in most vertebrates (Figure 2), their derived precursor genes are also likely to be spatially separate. The frequency of gene conversion has been shown to be small for distant genes, especially when they are located on heterologous chromosomes [16, 17].

The "Deep Homology" of Caeruleins

Because *cck* and *gastrin* share a common ancestry, the independently evolved *caerulein* genes are still homologous ("deep homology" as in [18]). To characterize the nature of the *cck/gastrin* duplication and hence of the caeruleins' deep homology, we screened the flanking regions of the hormone loci in various vertebrates and constructed synteny maps for adjacent genes (Figure 2). In most vertebrates, *cck* and *gastrin* are situated on different chromosomes, but both have retained synteny with the same set of genes. *cck* genes are typically flanked upstream by *eif1b* and downstream by *trak1*, *ulk4*, and *cttnb1*. Likewise, *gastrin* genes are flanked upstream by *eif1* and downstream by *hap1*, *jup*, and *no55.* For *S. tropicalis*,

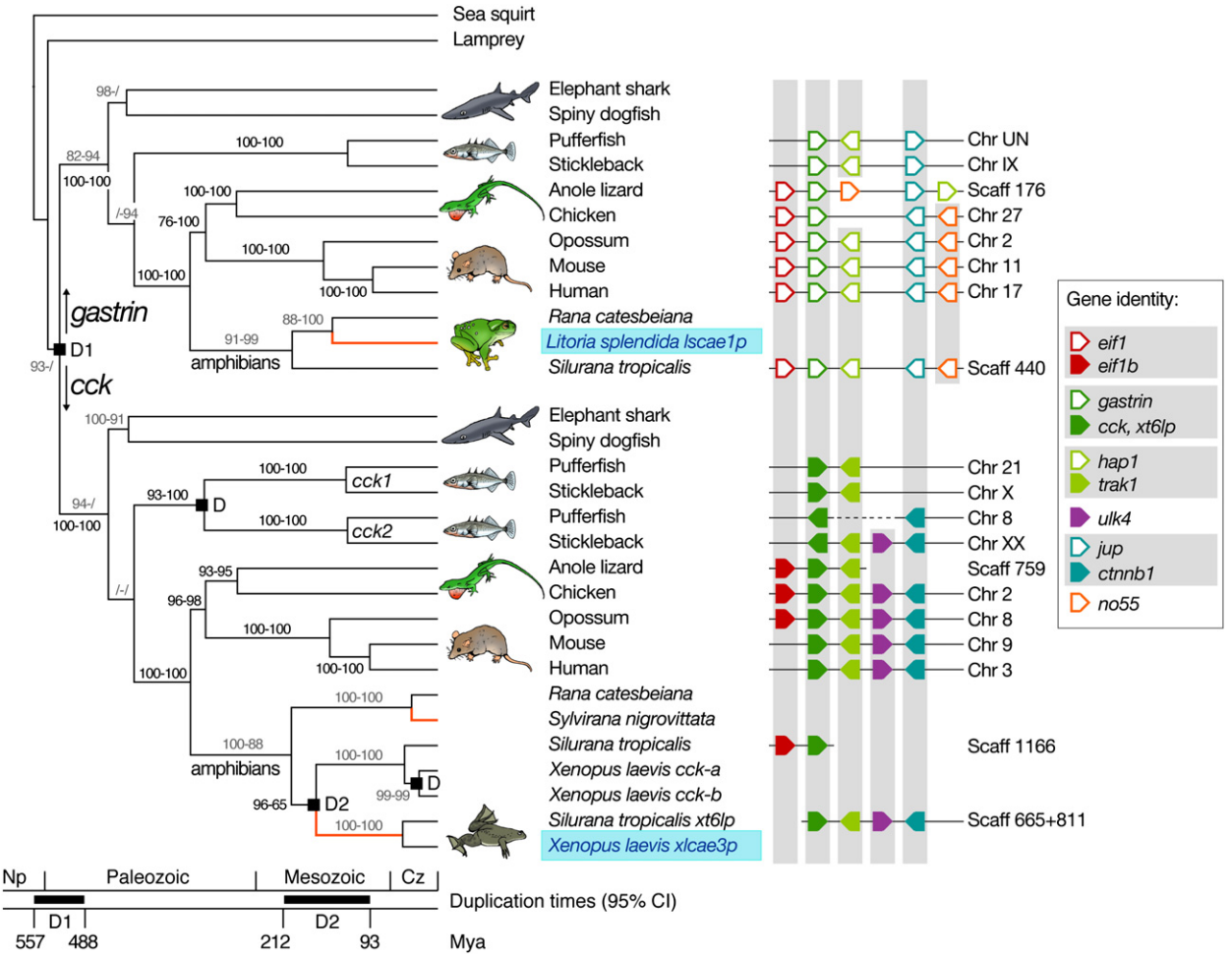

Figure 2. Phylogeny and Comparative Genomics of the Vertebrate *cck/gastrin* Gene Family

Left: parallel origins of caerulein are indicated in light blue. Inferred duplication events are labeled "D." Branch labels represent bootstrap percentages obtained by maximum parsimony (left) and maximum likelihood (right); values < 50% are indicated by a slash (/). Gray and black bootstrap percentages are calculated from data sets 1 and 2, respectively (see Results). The phylogeny is time calibrated with a Bayesian relaxed-clock model and priors for vertebrate divergence times derived from the fossil record and molecular studies (see Supplemental Experimental Procedures). Black horizontal bars in the timescale represent 95% credibility intervals for two relevant duplication events (nodes D1 and D2). Branches along which skin expression evolved are indicated in orange. Right: *cck* and *gastrin* genes of various vertebrates and *xt6lp* of *S. tropicalis* (filled and empty dark green symbols in the synteny maps) are flanked by homologous gene pairs (vertically aligned filled and empty symbols; see gene identity legend for color code), providing evidence for a segmental duplication (node D1) in the ancestor of jawed vertebrates. Depicted genes were included in data set 2. The following abbreviations are used: Np, Neoproterozoic; Cz, Cenozoic; CI, credibility interval; Mya, million years ago; Chr, chromosome; Scaff, scaffold.

synteny of *cck* (situated on genome scaffold 1166) with downstream genes could not be determined because of the limits of the available scaffolds. Instead, we found evidence for synteny of the *xt6lp* gene with *trak1*, *ulk4*, and *cttnb1* (by linking the partially overlapping scaffolds 665 and 811). Pairwise comparisons of the *cck*- and *gastrin*-flanking genes revealed homology between *eif1b* and *eif1*, between *trak1* and *hap1*, and between *cttnb1* and *jup*, respectively, indicating that they all arose by duplication. Together with their conserved synteny in jawed vertebrates, this finding indicates that at least four gene pairs, including *cck/gastrin*, originated by a single segmental duplication.

The conserved synteny of *cck* and *gastrin* with the other genes implies that their genealogical histories are correlated. This provided us with an expanded data set to consolidate the *cck/gastrin* tree and estimate the timing of relevant duplication events. A 10942 bp data set composed of *eif1/eif1b*, *cck/gastrin*, *trak1/hap1*, *ctnnb1/jup*, *no55*, and *ulk4* sequences (data set 2) resolved most nodes with high support (black bootstrap percentages in Figure 2). Relaxed molecular clock analyses [19] situate the *cck/gastrin* segmental duplication (node D1 in Figure 2) in the Cambrian period, approximately 557–488 million years ago (Mya) (95% credibility interval). The duplication event that produced the pipid *xt6lp/xlcae3p* ancestral gene (node D2) is estimated at 212–93 Mya. This interval encompasses time estimates for the split between Pipidae and Rhinophrynidae and for the basal diversification of Pipidae [20, 21], leaving open the possibility that related skin peptides are secreted by the single extant rhinophrynid species as well as other pipid genera.

Adaptive Evolution of *caerulein* Genes

Sequence convergence associated with recurrent origins of a new biological function can be considered one of the most

compelling indicators of molecular adaptation. We searched for signatures of natural selection in the peptide-encoding regions of the caerulein precursors. Likelihood-based estimation of the ratio of nonsynonymous over synonymous codon substitutions [22] revealed a ratio of 11.7 for the *xt6lp/xlcae3p* clade (indicating strong positive selection), compared to a background ratio of 0.03 in the remaining *cck* clade (indicating strong purifying selection). This result is significant according to a likelihood test for positive selection ($p = 0.0075$) and confirms that the origin of a skin-secretory function was accompanied by a major shift in selective pressure. The codons of the three residues that show convergence with *gastrin*-derived *lscae1p* (the two N-terminal Gln residues and Thr) bear high probabilities ($p \geq 0.999$) of positive selection. Similar analyses for *lscae1p* did not show evidence of positive selection, because the ratios for both the *lscae1p* branch and the remaining *gastrin* clade are estimated to be around 1.0 (suggesting neutral drift). This may be due to the fact that the frequency of nonsynonymous substitutions along the *lscae1p* branch hardly surpassed the background frequency along adjacent branches. Gastrin evolves at a faster rate than CCK [15, 23], which may be the result of relaxed selective constraints. In addition, sequence evolution along the *lscae1p* branch involved other types of mutations, which are not taken into account by codon models (including a codon insertion and C-terminal shortening). Nevertheless, our analyses suggest that both caeruleins originated independently under differing regimes of natural selection.

Besides convergence at the protein level, our results imply that *cck*- and *gastrin*-derived *caerulein* genes independently acquired transcriptional and posttranslational processing mechanisms for synthesis, storage, and secretion by skin glands. First, neither *cck* nor *gastrin* has been found to be expressed in the skins of *R. catesbeiana* and *X. laevis* [2, 23], suggesting that skin expression evolved fairly recently, after divergence from the closest known hormone relatives. The recent isolation of CCK from the skin of another frog, *Sylvirana nigrovittata* [24], suggests a third expression shift (Figure 2). Although the function of CCK in amphibian skin is unknown (it may represent the first evolutionary step of another toxin recruitment event), it demonstrates the propensity of this hormone family to evolve skin expression. Second, both caerulein precursors share a conserved Gly-Arg-Arg cleavage site at the C-terminal side of the decapeptide but different residues at the N-terminal side. In *Xenopus*, the N-terminal flanking sequence Arg-Arg-Phe-Ala-Asp-Gly has been postulated to be processed by an Arg-Arg cleavage followed by the stepwise removal of dipeptides [11]. The *Litoria* precursor, however, lacks an N-terminal Arg-Arg cleavage site.

Discussion

The advent of molecular phylogenetics has yielded numerous examples of evolutionary convergence, encompassing all domains of life and levels of biological organization. A major theme that has emerged during recent years is that similar phenotypes tend to evolve through different genetic mechanisms, but these often affect a single gene [25–30] or a small set of functionally related genes [31, 32]. These mechanisms range from selective shifts in extant allele variation [26, 28, 29] to new mutations in regulatory elements [25, 30] or coding sequences [27, 30, 32] and underlie recurrent adaptations among closely related populations [26–28, 30, 31] as well as different taxa [25, 29, 31]. Nevertheless, the full range of evolutionary processes generating recurrent phenotypes, and the extent to which adaptive convergence may be reflected in the underlying genetics, remains elusive. The present findings identify similar (convergent) mutations in peptides encoded by different (anciently duplicated) genes as an additional mechanism through which recurrent adaptations can arise across large phylogenetic distances. Even if one argues that the frogs in question are relatively closely related, the genes that gave rise to identical caeruleins represent a much older divergence, spanning nearly all vertebrates. Gene duplication has been predicted to be a major potential factor in functional convergence [33]. Several toxins found in the venoms of distant animal taxa, for example, evolved from proteins of the same family that share an ancient domain structure [34]. Although this can result in strikingly similar bioactivities, structural resemblance between these toxins often remained limited to conserved domain scaffolds or bioactive binding sites (but see [35]). The presently observed pattern implies not only that anciently duplicated genes provide a template for recurrent functional shifts but that this may be accompanied by extreme structural convergence until long after their divergence.

Extensive convergence in protein-coding sequences among different taxa has been reported before [36, 37]. "Melittin-related peptides," for example, found in the skins of two Japanese frog species, are up to 78% identical to melittin, an antimicrobial peptide in honeybee venom, but their precursor sequences suggest independent origins [38, 39]. Nevertheless, even when different starting conditions are exposed to indistinguishably similar selective pressures, convergent evolution is unlikely to be perfect, and structural differences are the logical outcome of the historically contingent nature of evolution. Consequently, structural convergence has been predicted to reflect a limitation in the number of adaptive solutions [40]. Caeruleins therefore delineate the extent of evolutionary determinism to which molecular adaptation can lead.

A crucial precondition for the parallel origins of caerulein was the conservation of the C-terminal bioactive domain. First, this domain allowed the recurrent evolution of a new biological function (from metabolic regulation to passive antipredator defense) while preserving a similar underlying biochemistry (as an agonist of CCK receptors [5]). Second, as a result of its widespread evolutionary conservation, the domain allows the peptides to be effective against a wide range of vertebrate predators. This broad-scale effectiveness could explain why identical toxins arose in frogs that inhabit different geographic realms and ecological habitats, where they are likely to face different types of predators (African pipids are strictly aquatic, whereas Australian/Papuan *Litoria* frogs have a terrestrial and/or arboreal lifestyle). Third, it determined the necessary preexisting structure (the peptide-encoding region and flanking residues) on which subsequent adaptive mutations could act. This situation is analogous to observations of deep homology in gene regulatory networks underlying the development of complex morphological adaptations such as eyes and limbs that evolved independently in distant animal phyla [18]. Our findings suggest that the same evolutionary patterns scale down to single-gene molecular adaptations as well.

Supplemental Information

Supplemental Information includes Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.cub.2009.11.015.

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