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# A subdivided molecular architecture with separate features and stepwise emergence among proinsulin C-peptides

Michael Landreh [a], Linus J. Östberg [a,b], Hans Jörnvall [a,*]

[a] Division of Physiological Chemistry I, Department of Medical Biochemistry and Biophysics, Karolinska Institutet, S-171 77 Stockholm, Sweden
[b] Science for Life Laboratory, Department of Medical Biochemistry and Biophysics, Karolinska Institutet, S-171 77 Stockholm, Sweden

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ABSTRACT

The C-peptide of proinsulin exhibits multiple activities and several of the underlying molecular interactions are known. We recently showed that human C-peptide is sub-divided into a tripartite architecture and that the pattern, rather than the exact residue positions, is a characteristic feature. We have now analyzed 75 proinsulins, ranging from fish to human and find a limited co-evolution with insulin, but with many marked deviations. This suggests a complex relationship, in which not only insulin affects the evolution of C-peptide. A subdivided nature, however, is a characteristic feature among all C-peptides, with the N-terminal segment the one most conserved. This segment, ascribed chaperoning charge-interactions with insulin, suggests that the insulin interactions constitute a basic function, although largely shifting from Glu to Asp residues in C-peptides of lower life forms. A second conserved feature is a mid-segment with a high content of adjacent Pro and Gly residues, in mammalian C-peptides compatible with a turn structure, but with fewer and more distantly interspaced such residues in the non-mammalian forms, and even absent in several fish forms. However, this segment of coelacanth C-peptide possesses a unique Cys distribution, capable of forming a disulfide-stabilized turn. Finally, the C-terminal segment of mammalian C-peptides, ascribed a possible receptor-interacting function, is not really discernable in the sub-mammalian forms. Combined, these patterns suggest an evolutionary stepwise acquisition of the tripartite mammalian C-peptide molecule, with insulin-interaction being ancestral, various turn stabilizations apparently of intermediate emergence, and possible receptor-interaction the most recent addition.



## 1. Introduction

Bioactive peptides are a heterogeneous group of often small polypeptides that trigger physiological processes in response to external stimuli. Common to all bioactive peptides is the presence of structural elements that mediate molecular interactions with target molecules such as membrane receptors or transcription factors. These interactions have to be highly specific, and consequently rely on well-defined features in both the messenger and the target.

Proinsulin C-peptide, the major part of the connecting peptide, linking the A- and B-chains of insulin, has been shown to affect physiological processes at multiple levels [1]. Firstly, C-peptide is required for the proper folding of proinsulin [2,3] and for chaperone-like functions during insulin maturation and storage in the secretory granules of the pancreatic β-cells [4,5]. Secondly, C-peptide can be internalized into cells and imported into the nucleolus, where it affects histone acetylation and rRNA transcription and may promote proliferative processes [6,7]. Thirdly, it binds to membranes in a manner suggesting the presence of specific interactions with a G-protein coupled receptor (GPCR) [8]. An orphan GPCR was recently reported as a potential C-peptide receptor [9], but is still not confirmed.

However, the C-peptide sequence is much more variable than that of the adjoining insulin segments [10]. Multiple studies have reported sequence alignments of proinsulin sequences to illustrate the low sequence identity among C-peptides from different phyla compared to that of the insulin A- and B-chains. In mammals, glutamic acid residues at positions 3, 11 and 27 and glutamine residues at positions 6 and 31 have been highlighted as largely conserved [10–13]. Co-variation analyses between C-peptide and insulin suggest that Glu3 has co-evolved with Glu13 of the insulin B-chain [13]. These findings, together with the observation that mutants lacking the acidic N-terminal region of C-peptide lead to proinsulin aggregation [2], have led to the suggestion that

* Corresponding author.
*E-mail address:* Hans.Jornvall@ki.se (H. Jörnvall).

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C-peptide merely provides constraints for proper insulin folding and disulfide formation [14].

Given that the strong conservation of insulin indicates a common function in all organisms across the animal classes, it seems unlikely that C-peptide has an equally uniform function or single physiological importance. In this article, we report that C-peptide sequences exhibit a limited co-variability with insulin but contain several conserved elements. As these can be linked to specific functions [15], the sub-divided bioactivity of C-peptide is concluded to be a general feature with successive additions.

## 2. Materials and methods

Proinsulin sequences were retrieved on May 7th, 2014, through a BLAST search of the human proinsulin sequence using the web interface of the UniProt database and the hits were downloaded as a FASTA file. Sequences annotated as insulin-like growth factors or fragments, as well as non-annotated sequences from organisms with an annotated insulin sequence were removed from the data set. The resulting set of 75 sequences contained 42 mammalian proinsulins, of which 10 stem from primates and 17 from rodents (four rodents with two proinsulin copies). Furthermore, six avian, five amphibian, three reptilian, and 19 fish proinsulin sequences were included. All sequences are listed in Supplementary Table 1. Sequences were aligned using the Kalign 2.0.4 software [16] and analyzed using the UGene 1.13.2 software package [17] after removal of the N-terminal signaling peptides and the two dibasic cleavage sites. Phylogenetic trees were constructed using the PHYLIP 3.695 software [18] and bootstrapped with 100 replicates. Tanglegrams were calculated using the Dendroscope 3.2.10 software [19,20] and optimized manually by rotating tree nodes. Secondary structure predictions were conducted using the PsiPred algorithm [21]. Models of the tertiary structure of coelacanth proinsulin were built using the I-Tasser software [22].

## 3. Results

### 3.1. Overall sequence variations of C-peptide and insulin are not strictly correlated

To evaluate the degree of overall correlation between insulin and C-peptide, we have compared the sequence identity between human insulin and C-peptide to those of 74 organisms across a wide range of phyla (Supplementary Fig. 1). As already known, C-peptide displays a lower degree of sequence identity than insulin. However, while the sequence identities of insulin and C-peptide generally decrease with increasing evolutionary distance and thus co-evolve to some extent, we find that they do not do so in a correlated manner. Instead, conservation in one of these partners is no indicator of conservation in the other. While *e.g.* the insulin sequence of the rhesus macaque is identical to the human, its C-peptide only has a sequence identity of 63%. Yet, the closely related crab-eating macaque shares 100% of the human insulin and 97% of the human C-peptide sequences. On the other end of the spectrum, the degu rat and the guinea pig, known for exceptionally large deviations from other mammalian insulin sequences (62% and 67% identical to human insulin, respectively) [23], have retained a high sequence similarity in C-peptide (75% and 79% identical to human C-peptide), thus even greater than for insulin. A direct comparison of the phylogenetic trees of mammalian C-peptide and insulin sequences with the human form as reference (Supplementary Fig. 2) support these observations. Both peptides follow an overall similar evolutionary path, which places *e.g.* most primates and rodents in the corresponding regions of their respective trees, but do not follow a complete co-evolution, and in some cases elicit different patterns with great deviations in either direction instead (criss-crossing tanglegram lines in Supplementary Fig. 2).

### 3.2. Overall sequence conservation of C-peptide

To identify which C-peptide features are conserved, we have compared the sequences of all C-peptides in the data set. From a multiple sequence alignment, three distinct groups can be discerned: Mammalian C-peptides make up the first group, birds, reptiles, and amphibia the second, and fish the third group (Fig. 1).

Common to all C-peptide sequences but one (Atlantic hagfish) is the presence of an acidic residue at position 3, which in all except one case (Sand snake) is a glutamic acid in the first two groups and mostly an aspartic acid in the third group. Furthermore, other glutamic and aspartic acidic residues are commonly found at the positions corresponding to residues 1 and 27 of the human sequence (1 and 31 in the composite Fig. 1). The mammalian, amphibian, reptile and avian C-peptides also contain a glutamic acid residue at the position corresponding to position 11 of the human sequence (15 in Fig. 1). Mammalian C-peptides additionally contain a middle segment with a high amount of prolines and glycines, some of which are also present in the avian and fish groups. The C-terminal glutamine residue common to mammals is of irregular occurrence in the second group and absent in the fish group.

### 3.3. Conformational features of C-peptides

Next, we compared the secondary structures of the C-peptides in the data set to complement the distinction of three segments previously identified in human C-peptide [15]. Fig. 2A shows the consensus sequence of the mammalian C-peptides, as well as the percentage at each position with that residue conserved. In addition to the singe residue conservation points highlighted before, we find that the distribution of secondary structure propensities among the C-peptides has a more extended pattern with prominent structural features as summarized in Fig. 2B for the mammalian peptides. The high conservation of Glu11 is accompanied by a common propensity to form a short β-strand or extended conformation spanning residues 9–13. Approximately half the sequences are predicted with low confidence to possess a β-strand propensity around positions 22–26, but an enrichment of β-strand-forming residues is not observed in this area. In the middle segment, the exact residue positions are only moderately conserved. However, this segment uniformly contains residues with a high turn propensity (Supplementary Fig. 3), suggesting that the segment between residues 13 and 19 in mammalian C-peptides preferentially adopts a turn-like structure. Interestingly, the fish C-peptide sequence from coelacanth (*Latimeria chalumnae*) contains two cysteine/half-cystine residues at positions 11 and 16 (Fig. 3), which is a unique feature among all C-peptides in the data set. These residues can form a disulfide bridge, as shown by a homology model of coelacanth proinsulin based on the human proinsulin structure, leading to the placement of the two cysteines in close agreement with the location of the turn sequence in human C-peptide (Fig. 3) and hence to a fixed rather than flexible turn structure. The modern-day coelacanth has been termed a "living fossil" due to its morphological similarities to fossilized species from the coelacanth lineage, which can, however, not be interpreted as genomic conservation [24]. In an unrooted phylogenetic tree, coelacanth C-peptide is located between the mammalian and non-mammalian branches (Supplementary Fig. 4), which may hint at a complex but functional evolutionary history behind its disulfide-stabilized turn.

In the C-terminal pentapeptide encompassing residues 27–31, approximately 40% of the mammalian sequences are predicted to

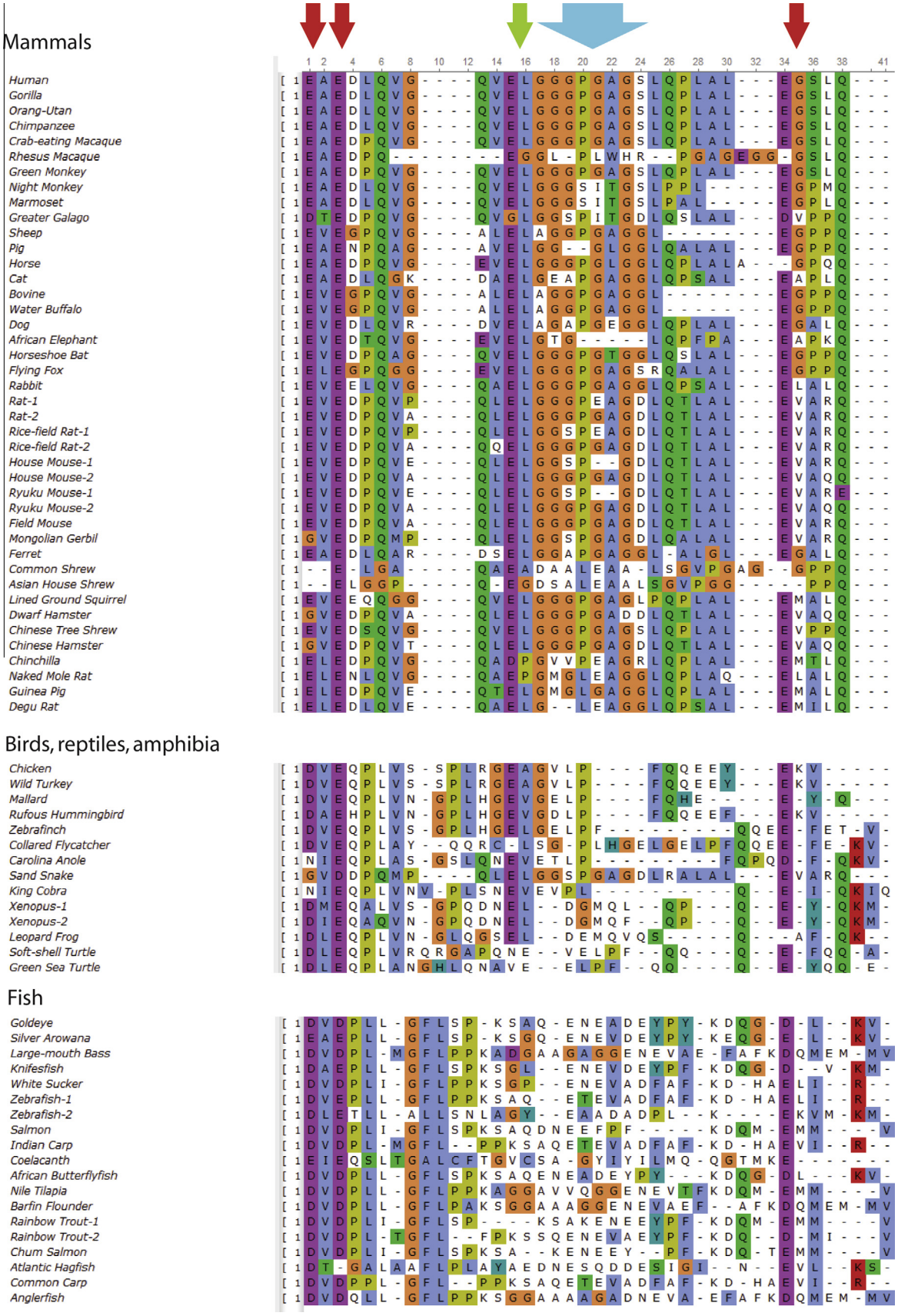


**Fig. 1.** Multiple sequence alignment of all C-peptides in the data set. Conserved acidic residues at positions 1, 3, and 27 of the human sequence are indicated by red arrows. The acidic residue at position 11 of the human sequence is indicated by a green arrow. The Pro/Gly-rich middle segment is indicated by a blue arrow (broad to illustrate the width). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

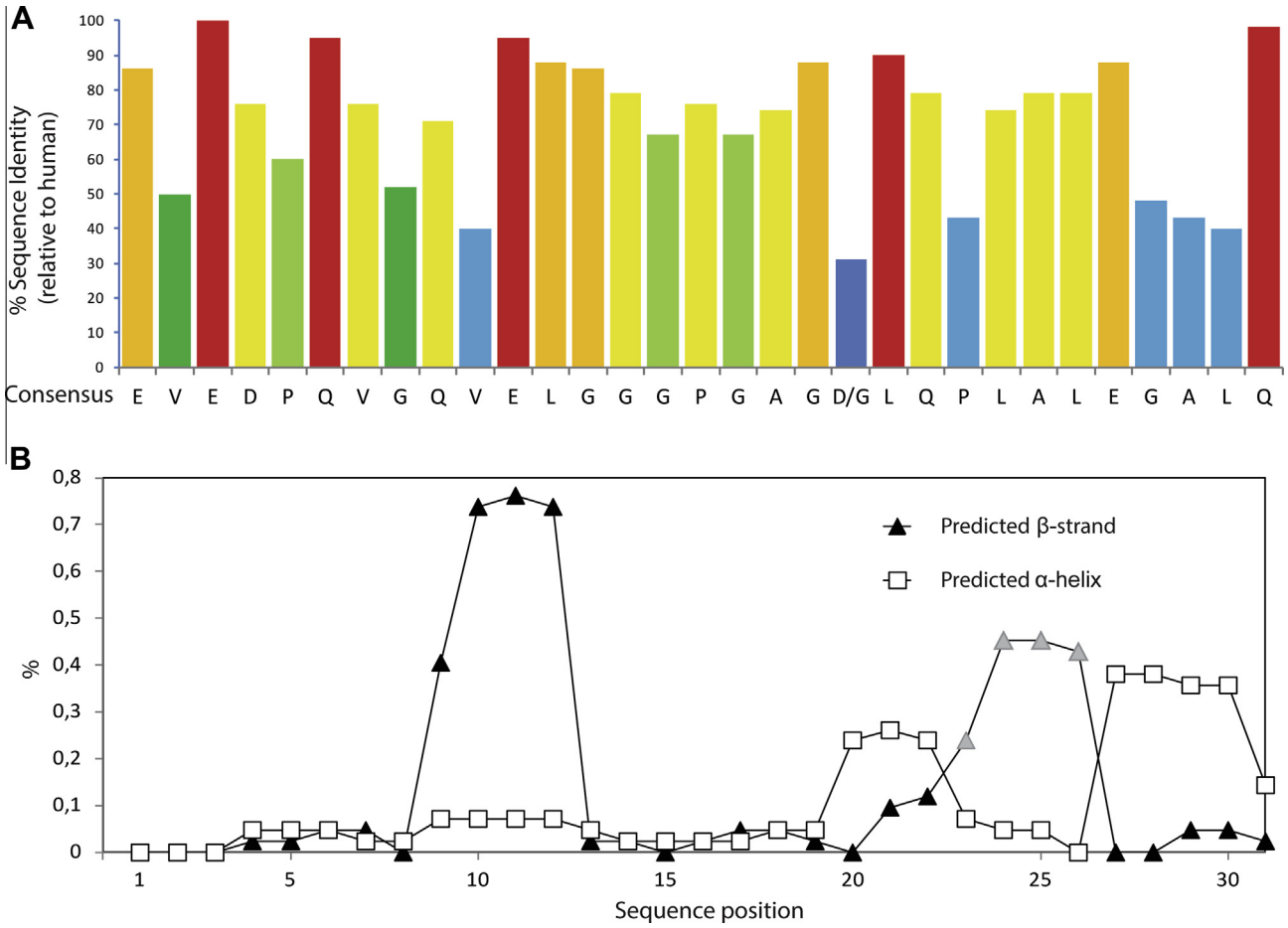


**Fig. 2.** Consensus sequence and secondary structure propensities of mammalian C-peptides. (A) A consensus sequence of the mammalian C-peptides based on the most abundant residue in each position shows that the glutamic acid at position 3 and the glutamine at position 31 are the best-conserved residues. Bar heights and coloring (red–blue) indicate the relative occurrence of each residue among all sequences. (B) Relative occurrence of secondary structure propensities among all mammalian C-peptides according to the PsiPred algorithm. The low-confidence β-strand propensity around residues 21–24 is indicated in gray. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

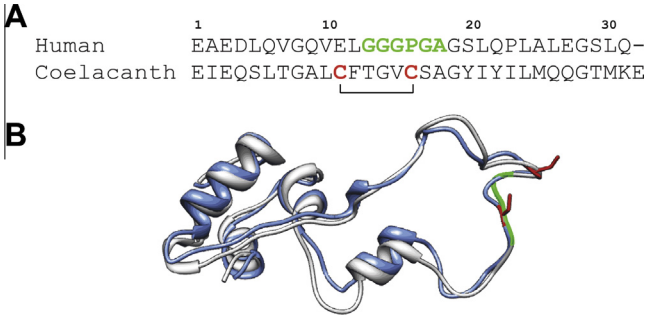


**Fig. 3.** Coelacanth C-peptide sequence and predicted structure. (A) Coelacanth C-peptide contains two half-cystines/cysteines at positions 11 and 16. (B) The predicted structure of coelacanth proinsulin, based on the human proinsulin structure (pdb ID 2KQP) shows that the cysteines (red) are located in the conserved turn region (green) of the mammalian C-peptides and could be oriented towards each other for disulfide formation. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

adopt a helical conformation. A comparison of the individual secondary structure preferences reveals that the C-terminal sequence EVARQ found in rodent C-peptides has a pronounced α-helical propensity. The sequence variant EGSLQ, which is common among primates, has a lower preference for helix formation, but can still adopt a helical conformation in solution [25]. The helical propensity, however, is absent in the sub-mammalian forms, as visible already by a rich presence of residues with a high β-strand preference in the fish group and to some extent also in the avian and reptilian groups. This residue distribution coupled with the lack of conservation separates the C-terminal segments of the two sub-mammalian groups from the mammalian C-peptides which have largely conserved pentapeptide end parts with a proposed receptor binding function [15].

Three structural elements, namely an extended conformation around residues 9–13, a flexible turn encompassing residues 14–18, and a C-terminal helix spanning positions 27–31, have been observed in molecular modeling and NMR studies of rat and human C-peptides [12,25–28]. Our results now demonstrate that, despite only moderate sequence conservation, a structurally conserved pattern exists (Fig. 4) among the mammalian, and to a lesser extent the non-mammalian C-peptides.

## 4. Discussion

We have demonstrated that C-peptide exhibits marked deviations from the evolutionary path of insulin, and that quite distinct structural features of C-peptide are conserved regardless of its high degree of sequence variation. The insulin and C-peptide co-production from a single proprotein implies a functional relationship [10]. Yet, the high variability of C-peptide compared

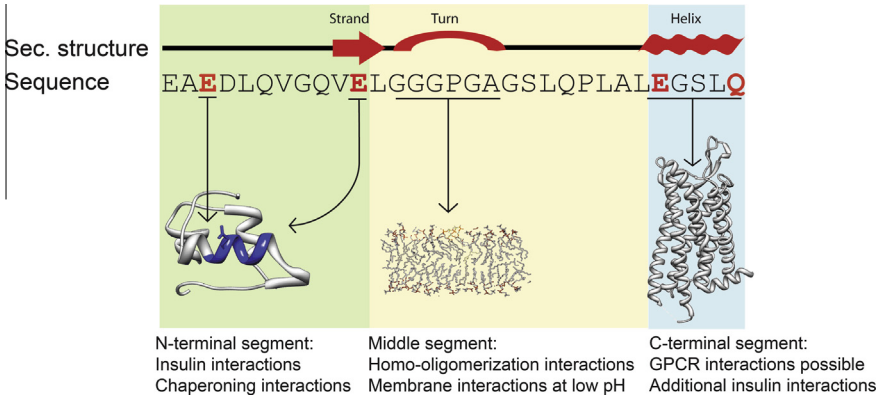


**Fig. 4.** The conserved features of C-peptide (highlighted in red) correlate with its tripartite architecture. They also correlate to some extent with biological activities, mainly from fragment studies, as indicated. The N-terminal segment (residues 1–11, green box) contains conserved Glu residues at positions 3 and 11 important in folding of the most aggregation-prone insulin segment (blue). The turn structure in the middle segment (residues 12–26, yellow box) mediates self-association and pH-dependent membrane interactions. The (pro-) helical C-terminal pentapeptide (residues 27–31, light blue box) contains a conserved glutamic acid and a conserved glutamine at positions 27 and 31, respectively, which have been implicated in GPCR activation. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

to insulin suggests that their relationship relies on a few defined features rather than the entire C-peptide sequence. This implies that C-peptide can have evolved also to meet requirements other than those imposed by its insulin partner. In line with this, each of the conserved C-peptide features described here can be assigned separate activities/functions [15], some which co-evolved with insulin and some which were acquired independently (Fig. 4).

The first conserved feature of C-peptide, the acidic N-terminal region, is related to the chaperoning activity of C-peptide on insulin. Chaperoning occurs in *cis* in proinsulin [2,3,14] as well as in *trans* between the mature peptides [4,29], and two well-conserved residues in C-peptide, Glu3 and Glu11, are required for these functions, respectively [2,29]. Furthermore, Glu3 in C-peptide has co-evolved with Glu13 of the insulin B-chain [13], located at the center of the most aggregation-prone insulin segment [30].

The second conserved feature of C-peptide constitutes the high content of prolines and glycines, concentrated in the middle segment of mammalian C-peptides, but more spread-out in the non-mammalian C-peptides and even absent in several of the fish forms. These residues convey considerable conformational flexibility and could fulfill multiple roles. In proinsulin, the tightly folded insulin moiety cannot accommodate the C-peptide part, and hence, the latter protrudes as a loop-like structure [31]. In this conformation, the flexible segments could act as a hinge between the two insulin chains during folding, or help to arrange the negative charges on C-peptide with the positively charged residues on insulin. If the coelacanth C-peptide is in fact disulfide-linked, the order of disulfide formation could provide a clue to whether the C-peptide or insulin segment acts as folding template. In addition, the flexible region has been implicated in C-peptide self-assembly and membrane interactions at low pH [28,32], both of which are accompanied by an increase in β-sheet content. Considering the β-strand propensity on the N-terminal side of the turn region in the mammalian C-peptides (Fig. 2B) [12,26], it appears possible that these parts adopt a hairpin-like structure.

The third partly conserved feature in the higher life forms is the C-terminal pentapeptide region of C-peptide. It has been suggested that this segment contains an interaction site for the binding to a G-protein coupled receptor (GPCR) [33]. GPCR activation has been related to the presence of an acidic residue at position 27 of the human sequence [34], which we now find is conserved among most C-peptides (position 34 in Fig. 1). In mammalian C-peptides, the relatively common helical propensity in this region and the presence of a second conserved residue at the C-terminus are in principle compatible with such an interaction [15], but absent in non-mammalian forms. It has recently been shown that the pro-helical carboxyterminal pentapeptide segment of rat neurotensin is sufficient to specifically bind and activate NTSR1 [35], a GPCR of the same family as the proposed C-peptide receptor GPR-146 [9]. However, such an interaction has not yet been investigated in detail. In summary, we have shown that C-peptides contain characteristic primary and secondary structural features that can be connected to different biological functions. Our observations suggest that C-peptide may have evolved step-wise with insulin interaction the oldest, possible receptor interactions the youngest acquisition and overall in an irregular co-evolution with insulin.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.07.012.

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