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Research Article

Separate functional features of proinsulin C-peptide

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Abstract. Proinsulin C-peptide influences a number of physiological parameters in addition to its well-established role in the parent proinsulin molecule. It is of interest as a candidate for future co-replacement therapy with insulin for patients with diabetes mellitus type 1, but specific receptors have not been identified and additional correlation with functional effects is desirable. Based on comparisons of 22 mammalian proinsulin variants, we have constructed analogues for activity studies, choosing phosphorylation of mitogen-activated protein kinases (MAPKs) in Swiss 3T3 fibroblasts for functional measurements. In this manner, we find that effective phosphorylation of MAPKs is promoted by the presence of conserved glutamic acid residues at positions 3, 11 and 27 of C-peptide and by the presence of helix-promoting residues in the N-terminal segment. Previous findings have ascribed functional roles to the C-terminal pentapeptide segment, and all results combined therefore now show the importance of different segments, suggesting that C-peptide interactions are complex or multiple.

Key words. C-peptide; diabetes mellitus; mitogen-activated protein kinase (MAPK); peptide secondary structure; tripartite functional segment.

Preproinsulin forms proinsulin by removal of the signal peptide and is then cleaved into insulin and C-peptide, which are stored in secretory granules and co-released into the circulation [1]. While the importance of insulin in metabolism was recognized very early on, C-peptide has long been considered to provide mainly structures for correct proinsulin folding, disulphide bond formation and cleavage [1]. This view has gained support from a low degree of conservation of C-peptide primary structures [2]. However, attention has also been drawn to a bioactive role of C-peptide, and a body of data now shows that C-peptide exerts favourable effects on diabetes-induced, functional and structural changes of the kidneys and peripheral nerves [3–9]. Patients with type 1 diabetes mellitus are hypothesised to need not only insulin but also C-peptide replacement therapy, and lack of C-peptide may be a factor behind the development of long-term complications in type 1 diabetes [9].

The mechanisms underlying the beneficial effects of C-peptide are enigmatic. A number of effects have been reported, including stimulation of phosphorylation of mitogen-activated protein kinases (MAPKs) in mouse embryonic fibroblast Swiss 3T3 cells [10], in mouse lung

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Table 1. Peptides studied for induction of ERK-1/2 phosphorylation.

Peptide	Sequence
C-peptide	EAEDLQVGQV ELGGGPGAGS LQPLALEGSL Q
E3A	EA**A**DLQVGQV ELGGGPGAGS LQPLALEGSL Q
E11A	EAEDLQVGQV **A**LGGGPGAGS LQPLALEGSL Q
E27A	EAEDLQVGQV ELGGGPGAGS LQPLAL**A**GSL Q
E3A/E11A/E27A	EA**A**DLQVGQV **A**LGGGPGAGS LQPLAL**A**GSL Q
D4A/V7A/G8A/V10A	EAE**A**LQ**AA**Q**A** ELGGGPGAGS LQPLALEGSL Q
D4P/V7P/G8P/V10P	EAE**P**LQ**PP**Q**P** ELGGGPGAGS LQPLALEGSL Q

Replacements versus C-peptide are in bold

capillary endothelial cells [11] and in human renal tubular cells [12, 13]. Requirements for protein kinase C, phosphoinositide 3-kinase, pertussis toxin-sensitive G-protein [10, 12, 13], and other factors [14] have also been shown, as well as insulinomimetic effects [15]. Several studies suggest that C-peptide exerts its effects through a G-protein-coupled receptor [16–18], but other receptors have also been implied [8], as well as mediation via non-chiral mechanisms [14]. The structure of C-peptide is unordered under physiological conditions, while the N-terminal part (residues 1–11) can adopt an α-helical conformation in high concentrations of trifluoroethanol (TFE) [19]. Other evidence suggests that the C-terminal pentapeptide part of C-peptide has an activity similar to that of full-length C-peptide in displacing C-peptide bound to a cell surface [16], in molecular assays [17, 20] and in stimulating glucose utilisation in diabetic rats [21]. Glu27 seems to be important for binding to cell surfaces [22]. The N-terminal Glu residue of C-peptide has also been ascribed some role in receptor interactions [23].

Despite all these data ascribing biological effects to C-peptide, a hormonal role of C-peptide is not universally accepted. In part this is because C-peptide exhibits extensive species variability, a property atypical of structures in functional interactions. No single residue is strictly conserved among all known forms of C-peptide, although several are conserved among mammalian species. Not even apparently important residues, like Glu27, are strictly conserved. The lack of extensive conservation is in sharp contrast to the situation with insulin, in which 18 residues are conserved among investigated animals (33% residue identity). These properties make it relevant to scrutinise C-peptide variability further and to evaluate its structure-activity relationships. In this study we have constructed, based on the proinsulin species variability, novel C-peptide analogues with different structural features for measurements of stimulation of phosphorylation of ERK1/2 in Swiss 3T3 fibroblasts. The results suggest that the N-terminal segment of C-peptide is also functionally active. Combined, all data suggest that C-peptide has a tripartite functional structure in separate interactions.

Materials and methods

Peptides

Recombinantly produced human C-peptide (Schwarz Pharma, Monheim, Germany) was used. Synthetically produced C-peptide analogues, shown in table 1, were obtained from K. J. Ross-Petersen (Horsholm, Denmark). All peptides were purified to apparent homogeneity using reverse-phase HPLC. Species variants of proinsulin were assembled from databanks. The variants were aligned using the software CLUSTAL W [24].

Peptide circular dichroism spectra were recorded between 180 and 260 nm with intervals of 1 nm using a circular dichroism spectrometer AVIV Model 62 DS, for 50 µM of each peptide in 10 mM sodium phosphate (NaPi) buffer, pH 7, and 0, 20, 40, 60, 80 and 95% (v/v) TFE. Background spectra recorded under identical conditions were subtracted from peptide spectra, and molar ellipticities, [θ], were calculated.

Cells and assay

The mouse embryonic fibroblast cell line Swiss 3T3 was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany). Cell cultures were held at 37 °C, 5% CO_2, in Dulbecco's modified Eagle medium (DMEM; Gibco, Invitrogen, Carlsbad, CA) without sodium pyruvate, with pyridoxine HCl, with 4500 mg/l glucose, supplemented with 50 µg/ml gentamicin (Gibco) and 10% (v/v) fetal calf serum (FCS), and were split every 3–6 days using trypsin/EDTA.

Cells of passage 6–10 were seeded in six-well plates at a density of approximately 2×10^4 cells/cm^2. Following 72–96 h of growth, the confluent or nearly confluent cells were washed with 37 °C-tempered starvation medium, i.e. the same medium as for cultivation but without FCS. The cells were kept in the starvation medium for 2–4 h at 37 °C, 5% CO_2. The buffer in each well was then changed to 37 °C-tempered Hanks' salt buffer (Gibco) and kept at 37 °C. The cells in each well were stimulated with 1 nM peptide in Hanks' salt buffer (at least three wells per peptide and experiment) or just Hanks' salt buffer as control

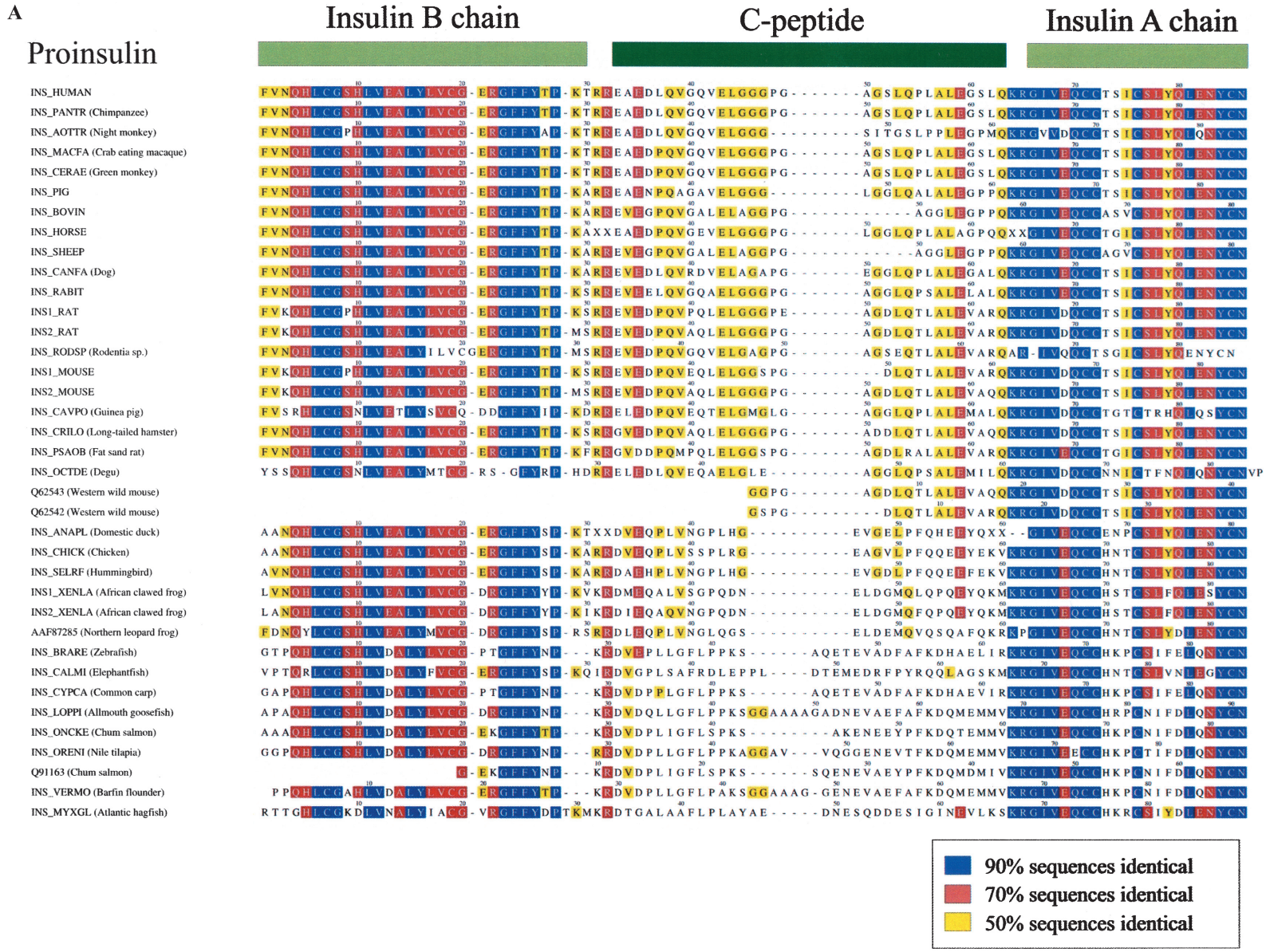

Figure 1. (*A*) Alignment of proinsulin primary structures, with colours indicating the extent of conservation at each position. Note conserved residues in C-peptide at either end, and mid-segment susceptibility to differences through both residue multiplicity and insertions/deletions.

B

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EAEDLQVGQVELGGGPGAGSLQPLALEGSLQ
GVdGP aPAL  AaSleLiGgsAGG aVPP
 L n  mAeA   ma  sdDerTSp  MAQ
   e   Edt   le  e t  lp   liR
       r                    m
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Figure 1. (*B*) Conservation of the 20 mammalian C-peptides from *A* is emphasized below the human C-peptide sequence (top line) by lower-case letters (residues present in one other species), capitals (residues present in more than one other species) and boxes (residues conserved in all or all but one mammalian species), while dashes (bottom) correspond to gap positions in at least one of the aligned mammalian C-peptides.

(at least three wells per experiment) for 3 min at 37 °C. The mode of pre-treatment followed previous schemes in measurements of MAPK stimulations [10–13], and the time involved was far shorter than $t_{1/2}$ for C-peptide degradation in tissue extracts [25]. Following peptide stimulation, the cells were rapidly washed with ice-cold Hanks' buffer, and lysed with lysis buffer, 50 mM HEPES (pH 7.5), 150 mM NaCl, 5 mM EDTA, 10 mM sodium pyrophosphate, 2 mM $NaVO_3$ (all from Sigma, St. Louis, MO), 1 × COMPLETE protease inhibitor (Boehringer Mannheim, Germany) and 1% (v/v) Nonidet P40 (USB, Cleveland, OH). The dishes with the cell lysates were kept on ice for 60 min, after which the lysates were removed with a rubber policeman, transferred to Eppendorf test tubes and homogenised by vigorous vortexing. The test tubes were kept on ice for 30 min, and were then vortexed again. After centrifugation (16,000 × g) at 4 °C, supernatants were transferred to new Eppendorf test tubes, kept on ice, and stored at –20 °C.

The protein content in each sample was determined using a BCA Protein Assay Reagent Kit (Pierce, Rockford, IL) and standardised by dilution of the sample with an appropriate amount of lysis buffer. The resulting samples were subjected to SDS/PAGE on 10% Tris/glycine gels or NuPAGE 10% Bis-Tris gels (both from Invitrogen), followed by blotting to 0.2 µm PVDF membranes (Invitrogen). The membranes were blocked in fat-free milk, washed, and incubated with primary antibody, rabbit anti-phospho-p44/42 MAP kinase (Thr202/Tyr204) antibody (Cell Signalling Technology, Beverly, MA). Duplicate membranes were incubated with a rabbit anti-[p44/42 MAPK] monoclonal antibody (Cell Signalling Technology) as a control of total MAPK levels. Following washing, the membranes were incubated with a secondary antibody, goat anti-rabbit IgG (H + L)-HRP conjugate (Bio-Rad, Hercules, CA), and washed again. Secondary antibodies were detected using an ECL Plus Western blotting detection system (Amersham Biosciences) and photographic film. The densities of the appropriate bands (reflecting phosphorylated p44/42 MAPK) on the resulting films were analysed using a Bio-Rad GS-710 calibrated imaging densitometer and accompanying Quantity One software.

Results of C-peptide stimulation of MAPK phosphorylation were analysed using a two-sided Student t test and the non-parametric Mann-Whitney U test. Differences with a p value below 0.05 in both tests were considered significant. p values in the Results section are from the Student t test. Data in the text and figures, presented as percentage stimulation of phosphorylation, are given as mean ± standard error.

Results

Conserved residues in proinsulin

The conservation pattern of 37 species variants of proinsulin, ranging from Atlantic hagfish to human, is shown in figure 1A. As already known, the insulin B and A chains are highly conserved, while C-peptide has only

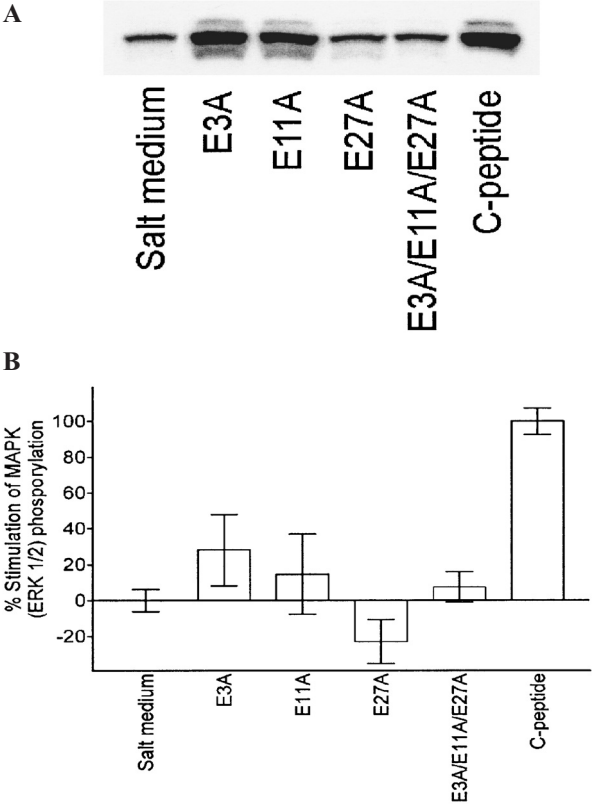

Figure 2. MAPK (ERK 1/2) phosphorylation patterns in 3T3 cells following treatment with C-peptide or Glu-substituted analogues. The cells were treated with 1 nM peptide or with salt medium. Individual Western blot gel patterns (*A*) and average (±SE) percentage stimulation (*B*) as obtained with anti-phospho-MAPK antibodies (n = 21 for the salt medium and n = 12 for each of all the peptides).

two residues (both Glu) highly conserved and exhibits internal deletions rendering also its length variable. However, considering mammalian C-peptides only, seven residues are strictly conserved or nearly so and can be subdivided into five one/two-residue parts, all with one Glu or Gln (fig. 1B). These parts cover the N-terminal segment (including Glu3, Gln6 and Glu11) and the C-terminal part (including Glu27 and Gln31). Residues of the intervening region are either variable, often Gly, Pro or further residues not favourable for secondary structure, or absent in some C-peptide species. In fact, any residue of the mid-part of C-peptide can be missing (fig. 1, positions 16–22 in *B*, corresponding in *A* to dashes in addition to those at the all-dash central segment).

The conservation pattern, with three well-conserved charges (Glu3, Glu11 and Glu27) and with some helix propensity in the N-terminal part [19], prompted us to test the importance of these features in C-peptide by measurement of stimulation of MAPK phosphorylation using C-peptide analogues with Glu to Ala replacements, with helix promoter (Ala) replacements, and with helix breaker (Pro) replacements.

Glu residues within C-peptide influence its capacity to phosphorylate MAPK

Human C-peptide produced a MAPK phosphorylation of 1.2–3.7 times the background phosphorylation (fig. 2), while synthetic analogues of C-peptide (table 1) in which Glu residues 3, 11 or 27 are replaced by Ala do not induce significant phosphorylation. The analogues substituted at position 3 (E3A) gave significantly less phosphorylation ($p < 0.01$) than C-peptide, as did the ones substituted at position 11 (E11A, $p < 0.01$), position 27 (E27A, $p < 0.001$) and positions 3, 11 and 27 (E3A/E11A/E27A, $p < 0.001$).

α-Helical propensity of the N-terminal part of C-peptide is correlated with its capacity to phosphorylate MAPK

Two synthetic C-peptide analogues, a tetra-Ala-substituted form (D4A/V7A/G8A/V10A C-peptide) and a corresponding tetra-Pro-substituted form (D4P/V7P/G8P/V10P C-peptide) (table 1), were designed to test varying propensities for α-helical secondary structure in the N-terminal part of C-peptide. As expected, CD spectra for the peptides in 0–95% TFE (fig. 3) showed that the three peptides differ regarding α-helical tendency in TFE. The tetra-Pro-substituted analogue showed virtually no sign of α-helical structure (no double minima at 208 and 222 nm), even in the presence of high concentrations of TFE, while both C-peptide and the tetra-Ala-substituted analogue showed spectra typical of helical peptides in the presence of TFE and also exhibited an isodichroic point at 203 nm, indicative of a two-state coil/helix behaviour. The spectra of the tetra-Pro-substituted analogue lacked such a point.

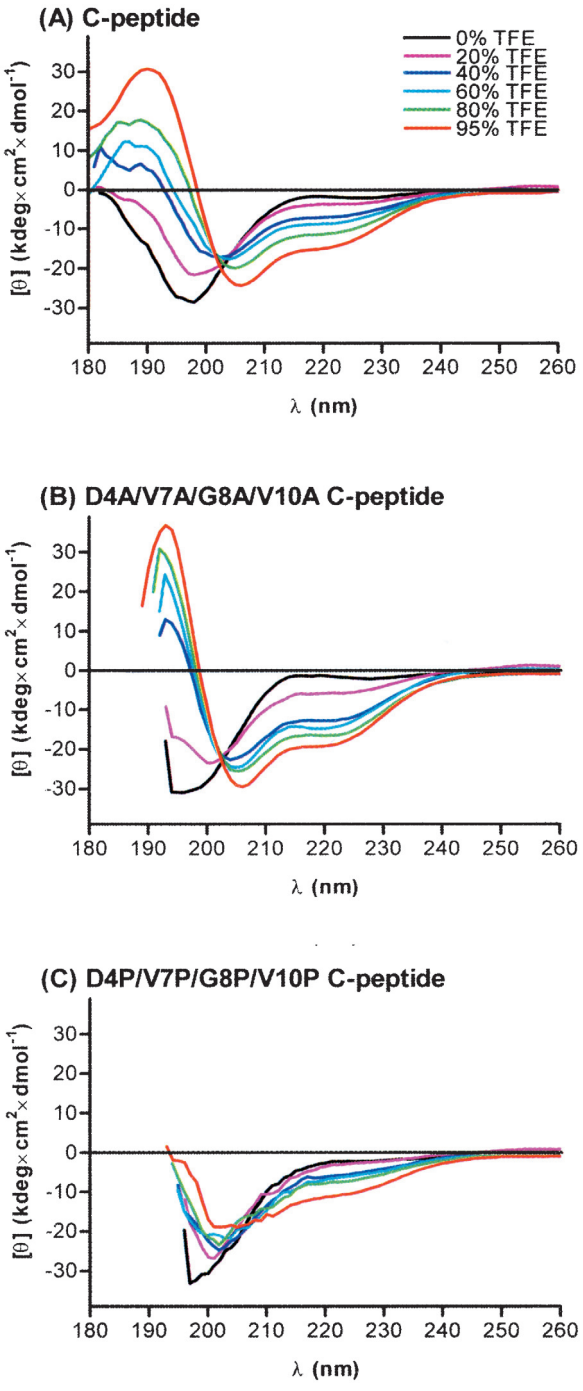

Figure 3. CD spectra for C-peptide and C-peptide analogues (50 µM) in sodium phosphate buffer, pH 7, with 0–95% TFE.

The propensity of each peptide for α-helical formation, as estimated by CD spectroscopy in TFE (fig. 3), was correlated with the capacity to induce phosphorylation of MAPK in 3T3 cells (fig. 4). The tetra-Ala-substituted analogue gave phosphorylation like the native peptide, significantly higher than for salt medium ($p < 0.01$). In contrast, the corresponding tetra-Pro-substituted peptide did not stimulate phosphorylation ($p < 0.01$).

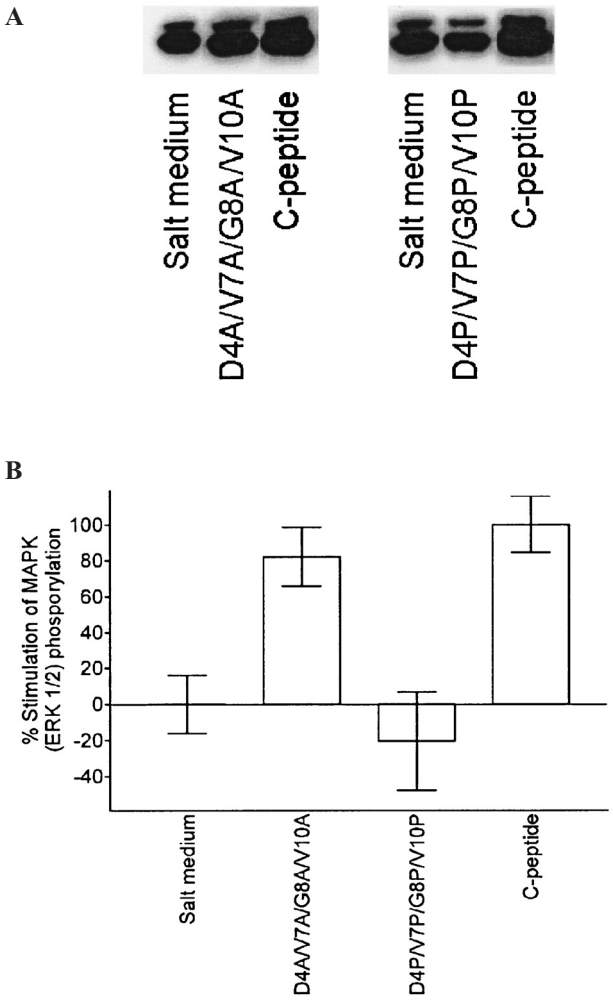

Figure 4. MAPK (ERK 1/2) phosphorylation patterns in 3T3 cells following treatment with C-peptide or the two helix-affecting analogues with Ala or Pro substitutions, at positions 4, 7, 8 and 10 (D4A/V7A/G8A/V10A and D4P/V7P/G8P/V10P). The cells were treated with 1 nM peptide or with salt medium. Individual Western blot gel patterns (*A*) and average (±SE) percentage stimulation (*B*) as obtained with anti-phospho-MAPK antibodies (n = 12 for the salt medium, n=9 for C-peptide, and n = 12 for the analogues).

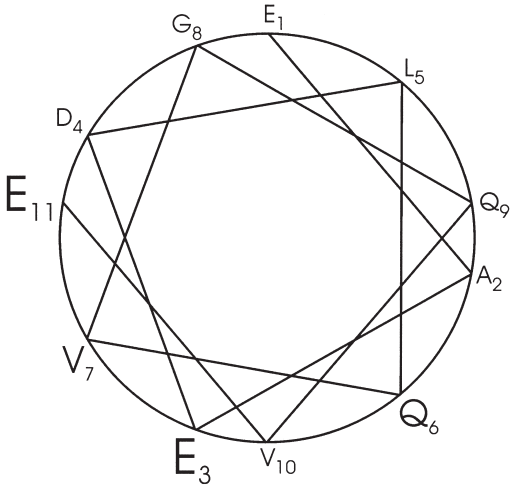

Figure 5. α-Helical wheel presentation of the N-terminal part of C-peptide. As shown, Glu3 and Glu11 (correlating with MAPK phosphorylation, fig. 2), and Gln6 and Val7 (conserved, or largely so, fig. 1B) would be adjacent (highlighted by increased letter size) in an induced helix.

Discussion

While C-peptide is clearly less conserved than insulin, it does show some conserved patterns among mammalian species in agreement with previous observations [2] and is not unique in variability among peptide hormones. The presence of several Gly, Pro and gap positions in the mid-C-peptide segment (fig. 1) suggests that the mid-C-peptide segment lacks secondary structure, or can be missing in part. Surrounding the mid-C-peptide segment, the two terminal segments contain six Glu/Gln residues and have been ascribed experimentally verified interactions. The N-terminal segment assumes an α-helix in TFE (fig. 3) and has the potential for a regular structure, not observed under physiological conditions [19], while the C-terminal segment exhibits binding interactions and biological activity [16, 17, 20].

Glu3, Glu11 and Glu27 are well conserved in mammalian species, as are Gln6, Gln31, Leu12, Leu26 and to some extent Glu1 and Val7 (fig. 1). Of these residues, the terminal positions (Glu1 and Gln31) line the proteolytic processing sites of proinsulin, while the codons for Gln6 and Val7 line an exon/intron junction in the gene [26], both facts which could explain these conservations. The remaining conserved positions are likely unaltered because of functional roles in C-peptide, and as now shown, the capacity to induce phosphorylation of MAPKs requires the presence of the conserved Glu residues (fig. 2). Glu27 is not only involved in C-peptide cellular membrane interactions and Ca^{2+} responses [19, 21] but is also required for induction of phosphorylation of ERK1/2. Similarly, lack of Glu at positions 3 and 11 results in loss of C-peptide stimulation of ERK1/2 phosphorylation.

Regarding the N-terminal α-helix propensity, one analogue (Pro-substituted) exhibits a low propensity and the other (Ala-substituted) a high propensity for α-helical formation, as verified by CD spectroscopy in aqueous TFE (fig. 3). The latter stimulates MAPK phosphorylation in the same range as native C-peptide, while the helix-destabilising, Pro-rich analogue does not stimulate MAPK phosphorylation. Thus, the propensity of C-peptide to form an N-terminal α helix (fig. 3) correlates with the capacity to induce stimulation of MAPK phosphorylation in 3T3 cells. Of course, other helix-destabilising analogues might have been synthesised, like Gly-substituted analogues. However, the C-peptide structure is unordered in aqueous solution [19], and the type of destabilisation therefore seems to be of little importance. Furthermore, the central C-peptide region is Gly rich and additional

multi-Gly substitutions might have promoted special poly-Gly folding structures. Hence, we chose Pro for the helix destabilisation. The nature of the residues at positions 4, 7, 8 and 10 is concluded not to be critical for C-peptide stimulation of MAPK phosphorylation, as long as they are compatible with helix formation.

Assuming that an N-terminal α-helical conformation is involved in C-peptide interactions, we note that both Glu3 and Glu11 are located at the same helix side as the conserved Gln6 and Val7 (fig. 5). Accompanied by the conservation pattern (fig. 1), this suggests a tripartite nature of C-peptide, in which the terminal sections, including the conserved Glu residues and specific secondary structures, may be involved in functional interactions, while the variable mid-section appears to form a joining segment. These results extend knowledge about critical residues in C-peptide and suggest that for total effects in cellular systems, the entire C-peptide is important, which is compatible with complex or multiple effects of C-peptide.

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1 Dodson G. and Steiner D.F. (1998) The role of assembly in insulin's biosynthesis. Curr. Opinion Struct. Biol. **8:** 189–194
2 Steiner D. F. (1984) The biosynthesis of insulin: generic, evolutionary and pathophysiologic aspects. In: The Harvey Lectures, series 78, pp. 191–198, Gotshlich E. C. (ed.), Academic Press, New York
3 Johansson B., Sjöberg S. and Wahren J. (1992) The influence of human C-peptide on renal function and glucose utilization in type 1 (insulin-dependent) diabetic patients. Diabetologia **35:** 121–128
4 Johansson B.-L., Borg K., Fernqvist-Forbes E., Kernell A., Odergren T. and Wahren J. (2000) Beneficial effects of C-peptide on incipient nephropathy and neuropathy in patients with type I diabetes. Diabet. Med. **17:** 181–189
5 Sima A. A., Zhang W., Sugimoto K., Henry D., Wahren J. and Grunberger G. (2001) C-peptide prevents and improves chronic type 1 diabetic polyneuropathy in the BB/Wor rat. Diabetologia **44:** 889–897
6 Samnegård B., Jacobson S., Jaremko G., Johansson B.-L. and Sjöquist M. (2001) Effects of C-peptide on glomerular and renal size and renal function in diabetic rats. Kidney Int. **60:** 1258–1265
7 Ekberg K., Brismar T., Johansson B.-L., Jonsson B., Lindström P. and Wahren J. (2003) Amelioration of sensory nerve dysfunction by C-peptide in patients with type 1 diabetes. Diabetes **52:** 536–541
8 Johansson J., Ekberg K., Shafqat J., Henriksson M., Chibalin A., Wahren J. et al. (2002) Molecular effects of proinsulin C-peptide. Biochem. Biophys. Res. Commun. **295:** 1035–1040
9 Wahren J., Ekberg K., Shafqat J., Johansson J., Johansson B.-L. and Jörnvall H. (2004) Biological effects of C-peptide and Proinsulin. In: International Textbook of Diabetes Mellitus and Proinsulin 3rd edn, pp. 165–181, DeFronzo R. A., Ferrannini E., Keen H., Zimmet P. (eds), Wiley, Chichester
10 Kitamura T., Kimura K., Jung B. D., Makondo K., Okamoto S., Canas X. et al. (2001) Proinsulin C-peptide rapidly stimulates mitogen-activated protein kinases in Swiss 3T3 fibroblasts: requirement of protein kinase C, phosphoinositide 3-kinase and pertussis toxin-sensitive G-protein. Biochem. J. **355:** 123–129
11 Kitamura T., Kimura K., Jung B. D., Makondo K., Sakane N., Yoshida T. et al. (2002) Proinsulin C-peptide activates cAMP response element-binding proteins through the p38 mitogen-activated protein kinase pathway in mouse lung capillary endothelial cells. Biochem. J. **366:** 737–744
12 Zhong Z. H., Davidescu A., Ehrén, I., Ekberg K., Jörnvall H., Wahren J. et al. (2005) C-peptide stimulates ERK1/2 and JNK MAP-kinases via activation of PKC in human renal tubular cells. Diabetologia **48:**187–197
13 Zhong Z. H., Kotova O., Davidescu A., Ehrén I., Ekberg K., Jörnvall H. et al. (2004) C-peptide stimulates Na^+,K^+-ATPase via activation of ERK1/2 MAP kinases in human renal tubular cells. Cell. Mol. Life Sci., **61:** 2782–2790
14 Ido Y., Vindigni A., Chang K., Stramm L., Chance R., Heath W. et al. (1997) Prevention of vascular and neural dysfunction in diabetic rats by C-peptide. Science **277:** 563–566
15 Grunberger G., Qiang X., Li Z., Mathews S. T., Sbrissa D., Shisheva A. et al. (2001) Molecular basis for the insulinomimetic effects of C-peptide. Diabetologia **44:** 1247–1257
16 Rigler R., Pramanik A., Jonasson P., Kratz G., Jansson O.T., Nygren P.-Å. et al. (1999) Specific binding of proinsulin C-peptide to human cell membranes. Proc. Natl. Acad. Sci. USA **96:** 13318–13323
17 Ohtomo Y., Bergman T., Johansson B., Jörnvall H. and Wahren J. (1998) Differential effects of proinsulin C-peptide fragments on Na^+,K^+-ATPase activity of renal tubular segments. Diabetologia **41:** 287–291
18 Li Z.G., Qiang X., Sima A. A. and Grunberger G. (2001) C-peptide attenuates protein tyrosine phosphatase activity and enhances glycogen synthesis in L6 myoblasts. Biochem. Biophys. Res. Commun. **280:** 615–619
19 Henriksson M., Shafqat J., Liepinsh E., Tally M., Wahren J., Jörnvall H. et al. (2000) Unordered structure of proinsulin C-peptide in aqueous solution and in the presence of lipid vesicles. Cell. Mol. Life Sci., **57:** 337–342
20 Shafqat J., Juntti-Berggren L., Zhong Z., Ekberg K., Kohler M., Berggren P.-O. et al. (2002) Proinsulin C-peptide and its analogues induce intracellular Ca^{2+} increases in human renal tubular cells. Cell. Mol. Life Sci. **59:** 1185–1189
21 Sato Y., Oshida Y., Han Y.-Q., Li L., Ekberg K., Jörnvall H. et al. (2004) C-peptide fragments stimulate glucose utilization in diabetic rats. Cell. Mol. Life Sci. **61:** 727–732
22 Pramanik A., Ekberg K., Zhong Z., Shafqat J., Henriksson M., Jansson O. et al. (2001) C-peptide binding to human cell membranes: importance of Glu27. Biochem. Biophys. Res. Commun. **284:** 94–98
23 Bourguignon J. P., Alvarez Gonzalez M. L., Gerard A. and Franchimont P. (1994) Gonadotropin releasing hormone inhibitory autofeedback by subproducts antagonist at N-methyl-D-aspartate receptors: a model of autocrine regulation of peptide secretion. Endocrinology **134:** 1589–1592
24 Thompson J. D., Higgins D. G. and Gibson T. J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. **22:** 4673–4680
25 Melles E., Jörnvall H., Tryggvason S., Ekberg K., Tryggvason K., Wahren J. and Bergman T. (2004) Degradation of proinsulin C-peptide in kidney and placenta extracts by a specific endoprotease activity. Cell. Mol. Life Sci. **61:** 2979–2982
26 Pictet R. L., Rutter W. J., Cordell B., Tischer E., Goodman H. M. and Bell G.I. (1980) Nucleotide sequence of a cDNA clone encoding human preproinsulin. Nature **284:** 26–32