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Research Article

Proinsulin C-peptide and insulin: Limited pattern similarities of interest in inter-peptide interactions but no C-peptide effect on insulin and IGF-1 receptor signaling

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Abstract. The recently reported influence of proinsulin C-peptide on insulin prompted us to examine structural features of the C-peptide. Four sets of limited pattern similarities towards inter-chain end regions of insulin were noticed, involving secondary structure elements, binding residues and intra- as well as inter-peptide residue similarities. Using surface plasmon resonance, we examined insulin binding to truncated, soluble insulin receptor A and IGF-1 receptor, but C-peptide effects on these bindings were not detectable. Two forms of the insulin receptor, differing in activation of gene transcription with regards to (pre)proinsulin and glucokinase, respectively, were also uninfluenced by C-peptide. We conclude that the pattern similarities, if functional, reflect C-peptide interactions with molecules other than both insulin A and B receptors and IGF-1 receptors. Any such effects are of interest in relation to reported binding interactions between insulin and C-peptide.

Keywords. Proinsulin C-peptide, insulin receptor, IGF-1 receptor, surface plasmon resonance, insulin/C-peptide segment.

Introduction

It has since long been established that a function of proinsulin C-peptide is to enable the correct folding and processing of proinsulin, but evidence now suggests further physiological roles of C-peptide. Disaggregation of insulin hexamers [1] is the most recently deduced factor in C-peptide function, presumably important in solubilization and secretion of pancreatic storage granulae in healthy subjects and of deposits at insulin injection sites in diabetic patients. Data also suggest that C-peptide binds to a G-protein-coupled receptor [2, 3], may be involved in non-chiral membrane interactions [4], and has insulinomimetic effects [5–7].

Liver, muscle and fat, as well as the insulin-producing β-cell [8, 9], are targets for positive insulin action on metabolic and mitogenic events. Studies on insulin-stimulated gene transcription in β-cells has revealed an isoform-specific read-out for the two insulin receptor (IR) isoforms, IR-A and IR-B [10]. Secreted insulin stimulates the transcription of its own gene *via* signaling through IR-A, but needs signaling *via* IR-B to activate glucokinase gene transcription. We were therefore interested in determining whether C-peptide differently modulates the IR-mediated transcriptional activations of these genes.

We have now compared the structure of C-peptide with those of insulin and IGF-1, focusing on the amino acid

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residues involved in binding of insulin/IGF-1 to the receptors [11] and on those associated with function or secondary structure in C-peptide [12–15]. Based on several sets of limited similarities found between C-peptide and insulin, we considered it relevant to re-investigate the effects of C-peptide in relation to insulin and IGF-1 receptor binding in a system with the pure components. Notably, even a non-insulin-related protein, visfatin, has recently been reported to bind to the IR [16]. We therefore examined whether C-peptide influenced IR isoform-specific pathways in a β-cell line.

Materials and methods

Proteins, peptides and cells. The purified human IR ectodomain sIR-11 (subtype A) [17] was a kind gift from Dr. Jakob Brandt, Novo Nordisk, Denmark. Recombinant human IGF-1 receptor (extracellular domains) (IGF-1R) and IGF-1 were obtained from Sigma. Human insulin was prepared by gel filtration of commercially available insulin (Actrapid® from Novo Nordisk, Denmark) and human C-peptide similarly from preparations of recombinantly produced C-peptide (Creative Peptides AB, Sweden). Scrambled C-peptide, a peptide with the same composition as C-peptide but with random sequence, was obtained from Sigma Genosys, UK. The cell line HIT-T15, derived from Syrian hamster islet of Langerhans β-cells, was supplied by American Type Culture Collection and cultured in RPMI 1640 medium fully supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine and 10% fetal calf serum at 5% CO_2 and 37 °C.

Immobilization of sIR-11 and IGF-1R on CM5 sensor chips. Carboxymethylated dextran-coated (CM5) sensor chips were used for immobilization of sIR-11 and IGF-1R. Following activation with *N*-hydroxysuccinimide (NHS) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide HCl (EDC), 40 µg/ml sIR-11 or 20 µg/ml IGF-1R in 10 mM sodium acetate buffer, pH 5.0, was applied to a separate sensor chip flow path, which was deactivated using 1.0 M ethanolamine HCl, pH 8.5. Sensor chips and chemicals were obtained from Biacore AB, Sweden.

Surface plasmon resonance (SPR) measurements. A Biacore 3000 instrument was used with different concentrations (0.5 nM–21.9 µM) of peptides prepared in running buffer containing 10 mM Tris, pH 7.4, 150 mM NaCl, 0.005% (v/v) surfactant P20 (SP20), and injected over sensor chip surfaces at 10–30 µl/min at 25 °C. Responses, recorded in sensorgrams as resonance units (RU) *versus* time, were time-scale transformed, background-subtracted and evaluated using BIAevaluation 4.1 software (Biacore AB).

Assay of transcription from proinsulin and glucokinase promoters. Cells were grown on 24-mm glass cover-slips and lipofectamine-transfected overnight with a plasmid, prIns1DsRed.rβGKGFP, containing expression cassettes for both rat (pre)proinsulin-1 promoter-driven red fluorescent protein (DsRed2) and rat glucokinase promoter-driven green fluorescent protein (GFP) [18]. After 24 h of culture, the cells were stimulated for 5 min with 0.3 nM insulin or 30 nM C-peptide, in fully supplemented RPMI 1640 culture medium containing 0.1 mM glucose. For co-stimulation, cells were preincubated for 20 min with 0.3–300 nM C-peptide in medium as above, to which 0.3 nM insulin was added for 5 min.

On-line monitoring of GFP and DsRed2 expression. Expressions were detected using digital imaging fluorescence microscopy [10, 19]. Glass cover-slips with transfected cells were placed in a perifusion chamber, mounted on an inverted microscope (Zeiss Axiovert 133TV; Carl Zeiss MicroImaging, US) equipped with a Zeiss plan NEOFLUAR ×25/0.8 Imm Korr lens (Carl Zeiss MicroImaging), kept at 37 °C and perifused with fully supplemented RPMI 1640 culture medium at 0.1 mM glucose. Excitation light was obtained from a SPEX fluorolog-2 MM1T11I spectrofluorometer (Spex Industries, USA). For GFP detection, excitation was at 485 nm, with a 505-nm dicroic mirror and a 505–535-nm band-pass emission filter; for DsRed2 excitation at 558 nm, a 565-nm dichroic mirror and a 580–620-nm band pass emission filter were used. Fluorescence was imaged using a cooled CCD camera (CH250 with KAF 1400; Photometrics, USA) connected to an imaging system (Inovision, USA). On-line monitoring was initiated 60 min after insulin/C-peptide stimulation, with cells chosen in 6–12 fields of view containing at least nine cells. The fluorescence intensity of each cell was calculated relative to its initial intensity, using the Isee software for UNIX (Inovision, USA).

Results

Pattern similarities between C-peptide and binding regions of insulin. The C-terminal part of the insulin B-chain and the N-terminal part of the insulin A-chain, *i.e.* the two-part insulin segment surrounding the C-peptide location in proinsulin, are structured and proximate in folded insulin. Four patterns in these segments appear similar to those in C-peptide (Fig. 1). First, several of these positions exhibit inter-peptide identical residues between insulin and C-peptide. Second, several of the residues within this two-part insulin segment and C-peptide are also conserved within the peptides among mammalian species. Third, several of the conserved residues in insulin are involved in binding to the IR [20] (and for the homologous IGF-1 in binding to the IGF-1R [21])

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            hhhhhhhhhhh                  ttttt
              *     *                    *
C-peptide   EAEDLQVGQVELGGGPGAGSLQPLALEGSLQ
Insulin     LVEALYLVCGERGFFYTPKT    GIVEQCCT
             **  **   ** * *  *      **  *
            αααααααααααα βββ TT      αααααTβ
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Figure 1. Segment of human C-peptide (upper sequence) and human insulin B-chain residues 11–30 followed by A-chain residues 1–8 (lower sequence), with conserved residues in 95% or more of the mammalian species in bold. Boxed residues are identical between C-peptide and insulin (and identical also with IGF-1 and IGF-2, except for Asp instead of Glu at B13 in both IGF-1 and -2, and at A4 in IGF-1. Asterisks below the insulin sequence indicate residues that are concluded to contact the IR [11], whereas asterisks above the C-peptide sequence indicate functionally important residues [12, 13]. The bottom line indicates residues in α-helical (α), β-strand (β) or turn (T) structures of insulin (Swiss-Prot entry P01308), and the uppermost line indicates residues with propensities for helical (h) or type III' β-turn (t) structures of C-peptide [14, 15].

and several of those in C-peptide are also associated with known binding functions (Glu residues, [12, 13]). Fourth, several of these residues reside in regions with secondary structure in both molecules. Thus, we observe multiple but limited similarities between a two-part insulin segment (B11–B30 plus A1–A8), the corresponding segment in the related IGF-1 molecule, and the entire C-peptide (Fig. 1). Although the whole segment is, of course, short and the similarities are limited, the coincidence of three critical Glu residues [12] involved in binding interactions is of particular interest, and no other alignment than that shown in Figure 1 gives this coincidence.

Effect of C-peptide on sIR-11 or IGF-1R. SPR sensorgrams for a wide range of C-peptide concentrations (0.5 nM–21.9 μM) over immobilized sIR-11 and IGF-1R were recorded. In the sensorgram (Fig. 2a), showing the response of an sIR-11 surface to different concentrations of C-peptide, the resulting curves lack typical association and dissociation phases and exhibit low maximal values. The response curves of IGF-1R surfaces to different concentrations of C-peptide were very similar (not shown). Furthermore, scrambled C-peptide (0.5 nM–5 μM) also yielded similar sensorgrams to those for C-peptide to both receptors (not shown).

Effect of C-peptide on binding of insulin to sIR-11 and IGF-1R. The sensorgram of Figure 2b shows the response of an sIR-11 surface to different concentrations of insulin, illustrating that this low-affinity receptor retained its binding capacity following immobilization to and mild regeneration of the sensor chip surface. A corresponding sensorgram for an IGF-1R surface was very similar to that in Figure 2b. The binding of insulin was not much affected by the presence of C-peptide in equimolar or higher (1–10×) concentrations. As shown in the sensorgram of Figure 2c, the response of the sIR-11 surface to insulin was shifted slightly upwards (but so is the response to C-peptide *per se*), while the association and dissociation patterns remained unchanged. The binding of insulin to IGF-1R was also not much altered in the presence of C-peptide, thus giving similar curves and shifts as with sIR-11 surfaces. Scrambled C-peptide (0.5 nM– 5 μM) shifted the insulin binding curves to sIR-11 and IGF-1R slightly in the same manner as observed for C-peptide, the shifts also largely corresponding to the response to scrambled C-peptide only.

Effect of C-peptide on binding of IGF-1 to IGF-1R. The sensorgram of Figure 3b shows the response of a functional IGF-1R surface to different concentrations

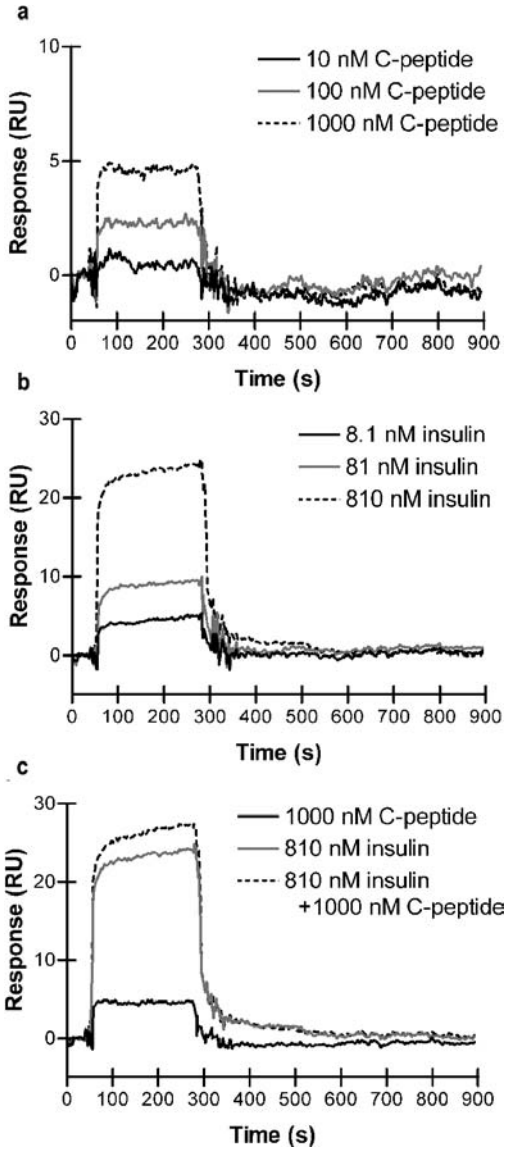

Figure 2. SPR sensorgrams of C-peptide (*a*), insulin (*b*) and a combination of insulin and C-peptide (*c*) injected for 4 min over a surface with immobilized sIR-11 at a flow of 10 μl/min.

of IGF-1. The dissociation of IGF-1 from this IGF-1R is considerably slower than the corresponding actions of insulin (Fig. 2). The binding of IGF-1 is not influenced by the presence of C-peptide (0.1–10× concentration) (Fig. 3c); the association and dissociation patterns remain intact.

Effect of C-peptide on IR-mediated transcription of the (pre)proinsulin and glucokinase genes in the pancreatic β-cell. To test whether C-peptide modulates IR-A-mediated activation of (pre)proinsulin gene transcription, IR-B-mediated activation of glucokinase gene transcription, or both, we analyzed the effect of C-peptide on (pre)proinsulin promoter-driven DsRed expression and glucokinase promoter-driven GFP expression in transfected HIT-T15 cells. Stimulation of HIT-T15 cells with 0.3 nM insulin at a substimulatory glucose concentration (0.1 mM) produced an approximately 1.6-fold increase of (pre)proinsulin gene transcription, and approximately 1.5-fold increase of glucokinase gene transcription compared to unstimulated control cells. As shown in Figure 4a, stimulation of the cells with 30 nM C-peptide did not alter the transcriptional levels of these two genes, compared to unstimulated control cells. The corresponding transcriptional levels resulting from stimulation of HIT-T15 cells with 0.3 nM insulin, following pre-treatment with 0.3–300 nM C-peptide (Fig. 4b), showed that C-peptide did not interfere with IR-A-mediated activation of the (pre)proinsulin promoter or IR-B-mediated activation of the glucokinase gene promoter in these cells.

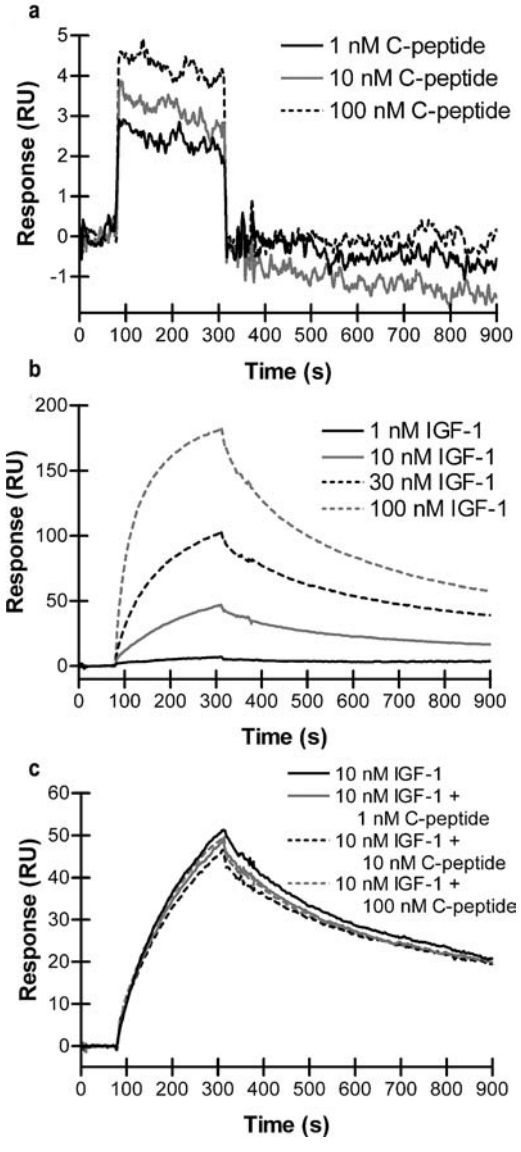

Figure 3. SPR sensorgrams of C-peptide (*a*), IGF-1 (*b*) and a combination of IGF-1 and C-peptide (*c*) injected for 4 min over a surface with immobilized IGF-1R at a flow of 10 µl/min.

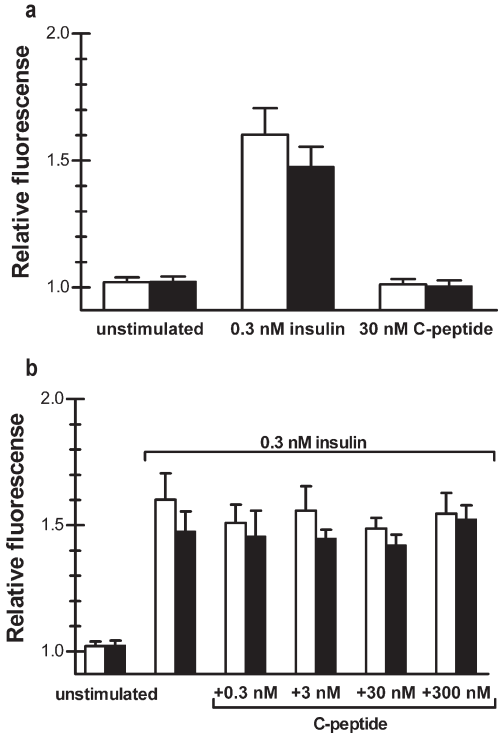

Figure 4. Transcriptional activities at the proinsulin (white) and glucokinase (black) gene promoters relative to corresponding activities in unstimulated HIT-T15 cells, following stimulation of the cells with (*a*) insulin or C-peptide, or (*b*) a combination of insulin and C-peptide. Indicated spreads show ±SE.

Discussion

In view of reports implicating interactions of C-peptide with insulin signaling and insulin disaggregation [1, 5–7, 22], we found it relevant to scan for similarities between C-peptide and receptor-binding regions of insulin (Fig. 1), and to re-investigate in a pure system whether C-peptide

binds to or influences binding to purified receptors for insulin or IGF-1. Unexpectedly, another protein, visfatin, has recently been found to bind to the IR, although in a manner distinct from that of insulin [16]. We found four pattern similarities between C-peptide and functionally important inter-chain end regions of insulin. If functional, they may reflect the C-peptide/insulin interaction region implicit in the insulin oligomer disaggregation effect of C-peptide [1]. Hence, apart from the previous data supporting C-peptide/oligomeric insulin binding, there may even be structural similarities compatible with such a site in insulin and C-peptide.

In spite of this positive finding, we still did not find evidence of any direct interaction between C-peptide and the insulin or IGF-1 receptors. Thus, truncated, soluble forms of the IR [17] and IGF-1R, encoding extracellular parts, coupled to a sensor chip surface allowing repeated experiments in a SPR biosensor, gave no evidence of a direct interaction of C-peptide with the IR (Fig. 2a) or the IGF-1R (Fig. 3a). In addition, the presence of C-peptide did not influence the association and dissociation of insulin (Fig. 2c) or IGF-1 (Fig. 3c) to these receptors, although the latter were of different patterns. It is unlikely that the slight increase in SPR signal observed in the presence of C-peptide (Fig. 2c) reflects an increased availability of insulin monomers [1], since scrambled C-peptide also gave this small increase. Notably, it is theoretically possible that receptor binding to the chip surface might block a C-peptide binding site, and that an alternative receptor assay might have been desired. Radioligand receptor assays for C-peptide binding are not available at present; however, although ligand binding can be noticeable with fluorescence correlation spectroscopy [2], a blocked peptide binding site is now also unlikely since the correct peptide ligands do bind to their receptors (Fig. 2 and 3).

We observed considerably slower dissociation of IGF-1 (Fig. 3b) than of insulin (curve similar to that in Fig. 2b) from IGF-1R. This probably reflects the low capacity of insulin to induce the conformational change of IGF-1R required for high-affinity binding [23]. Notably, the association and dissociation patterns of insulin to sIR-11 (Fig. 2b) and IGF-1R are quite similar. The reason may be that the sIR-11 construct has a fairly low affinity for insulin [21, 24]. These results extend observations that IR interactions can be studied using SPR methodology [25]. Glucose-induced changes of transcription of the (pre)proinsulin gene in rat pancreatic β-cell requires ERK1/2 activation [26]. C-peptide has been shown to increase ERK1/2 activity in mouse fibroblasts and lung capillary endothelial cells [12, 27, 28], rat aortic endothelial cells [29] and human renal tubular cells [30], and to reduce high glucose-induced phosphorylation of ERK1/2 (p42/p44 MAP kinase) in rat aortic smooth muscle cells [31]. The β-cell exhibits two distinct IR (IR-A and IR-B), which activate separate intracellular pathways [10]. Although C-peptide is implicated in intracellular pathways and produces a cellular Ca^{2+} influx [6, 7, 32–34], we did not observe C-peptide interaction with insulin signaling *via* IR-A or IR-B on β-cells (Fig. 4). This supports the notion that effects of C-peptide on insulin signaling involve other factors than the IRs themselves. In this regard, the segment of C-peptide/insulin/IGF-1 similarity may be one such factor promoting inter-peptide interactions.

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