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C-Peptide Binding to Human Cell Membranes: Importance of Glu27

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In addition to its established role in proinsulin folding, C-peptide has a function in regulation of cellular activity. The 31-residue peptide influences renal, vascular, and metabolic functions in patients with insulin-dependent diabetes mellitus. Binding to cells has been demonstrated for C-peptide, which can be displaced by its C-terminal pentapeptide. We have now used fluorescence correlation spectroscopy to investigate structural requirements on the pentapeptide part for C-peptide binding. All pentapeptide residues, $E_{27}GSLQ_{31}$, were individually replaced with Ala and the capacity of the resulting peptides to displace rhodamine-labelled full-length human C-peptide from human renal tubular cell membranes was determined. This showed that Glu27 is essential for displacement, while replacement of Gly28 with Ala has little effect, and replacement of any of the three most C-terminal residues had intermediate effects. Morevover, free Glu displaces full-length C-peptide to about 50%, while free Ala, C-peptide(1–26), and the truncated pentapeptide, corresponding to the tetrapeptide $G_{28}SLG_{31}$, have no displacing capacity. The peptides EVARQ (corresponding to the rat C-terminal pentapeptide) and ELGGGPGAG (corresponding to positions 11–19 of human C-peptide) do not displace human C-peptide. These results indicate that Glu27 of C-peptide is critically involved in binding to cellular targets. © 2001 Academic Press

Key Words: **C-peptide binding; fluorescence correlation spectroscopy; Ala-scan analysis; C-peptide pentapeptide fragment; glutamate receptor.**

Insulin is expressed in β-cells in the islets of Langerhans as preproinsulin, which is processed in several steps to mature insulin and to the 31-residue C-peptide, derived from the part originally connecting the insulin A and B chains (1, 2). Insulin and C-peptide are stored in secretory granules before their release to the blood circulation. Previously, it has been thought that C-peptide lacks cellular activity. However, C-peptide replacement in animals with experimental diabetes and in patients with type 1 diabetes is accompanied by improved renal function (3–5), increased glucose utilization (6–8), increased muscle and skin blood flow (9, 10), and improved autonomic and sensory nerve function (5, 11). C-peptide also improves erythrocyte deformability in type 1 diabetes patients (12) and inhibits leokocyte-endothelium interactions (13). Different mechanisms of action of C-peptide have been proposed. Stimulation of Na^+,K^+-ATPase and endothelial NO synthase activities, accomplished via activation of a G-protein coupled receptor and Ca^{2+}-dependent intracellular signalling pathways, have been observed in rat renal tubular segments (14) and aortic endothelial cells (15). In addition, C-peptide effects on albumin permeation mediated by direct peptide/membrane interactions, thus not involving a stereospecific receptor, have been reported (16). It was recently found that C-peptide crosstalk with insulin signaling at the level of the insulin receptor may be involved in stimulation of glycogen synthesis in myoblasts (17), and that C-peptide stimulates mitogen-activated protein kinases in Swiss 3T3 fibroblasts (18).

We could previously demonstrate stereospecific binding of C-peptide to intact human cells (19) and to solubilized cell membranes (20), using fluorescence correlation spectroscopy (FCS), while no C-peptide/lipid interactions could be observed by circular dichroism spectroscopy and size-exclusion chromatography (21). The binding of C-peptide to intact cells is displaceable

Abbreviations used: FCS, fluorescence correlation spectroscopy; RhCP, rhodamine-labelled C-peptide.

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both by full-length C-peptide and by a pentapeptide consisting of the five C-terminal amino acid residues of C-peptide (19). Furthermore, the stimulation of Na^+,K^+-ATPase activity by C-peptide can be mimicked by this C-terminal pentapeptide (22). Taken together, these effects suggest not only a physiological hormonal role of C-peptide, but also that the effects of C-peptide are to a great extent mediated via its C-terminal pentapeptide fragment.

We have now used an "alanine scan" approach, employing synthetic pentapeptides in which each of the five amino acid residues, one at a time, has been replaced with alanine. We have examined the extent to which the modified pentapeptides function as competitive inhibitors of the cellular binding of labelled C-peptide, using the FCS technique. We have also evaluated free glutamic acid as a displacer and correlated the results with species variants of C-peptide. The results show the relative importance of the five pentapeptide amino acid residues and focus particular attention on the importance of Glu27 of intact C-peptide for cellular binding.

MATERIALS AND METHODS

Cell culture. Human renal tubular cells were cultured from the unaffected outer cortex of renal tissue obtained from nondiabetic patients undergoing elective nephrectomy for renal cell carcinoma (23). The cells were cultured in RPMI 1640 (Life Technologies, Grand Island, NY) supplemented with 10% fetal calf serum, 2 mM glutamine, 10 mM Hepes, benzylpenicillin (100 units/ml), and streptomycin (100 mg/ml) and passaged at near confluence by trypsinization. Growing cells exhibited epithelial morphology with a central nucleus, a granular cytoplasm, and cobblestone appearance on light microscopy. Cells from the second and third passages were used for the experiments.

Peptides and amino acids. Human C-peptide, the peptides EGSLQ, AGSLQ, EASLQ, EGALQ, EGSAQ, EGSLA, GSLQ, EVARQ, ELGGGPGAG, rat C-peptide, and C-peptide(1–26) were synthesized by Sigma Genosys, Cambridge, with a purity of >95%. Porcine C-peptide was obtained from Linco, NY. L-Ala and L-Glu were purchased from Sigma.

Labelling of C-peptide. Human C-peptide was labelled with rhodamine (absorption 555 nm, emission 580 nm), using a succinimidyl ester derivative (FluoReporter, F-6163; Molecular Probes) and isolated as described earlier (19). The identities of the purified labelled peptides were confirmed by matrix-assisted laser desorption ionisation mass spectrometry.

Fluorescence correlation spectroscopy. FCS was performed as previously described (19). All binding studies were carried out on cells cultured in eight-well Nunc chambers (Nalge Nunc) at 20°C. Prior to the experiments, cells were washed five times with phosphate buffered saline and incubated with binding buffer [20 mM Hepes (pH 7.4), 115 mM NaCl, 24 mM $NaHCO_3$, 4.7 mM KCl, 1.26 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 11.1 mM glucose, 5 mg/ml bovine serum albumin]. Binding of C-peptide was measured by FCS after 60 min incubation of the cells with 5 nM rhodamine-labelled human C-peptide (RhCP). Competitive displacement of bound RhCP by either one of the peptides or amino acids was measured following incubation for 3 h (sufficient for maximal displacement) with 5 μM of the added peptides (postincubation). Displacement was calculated as: $(W_{CP} - W_D)/W_{CP}$; where W_{CP} is the sum of the weight factors of the components with diffusion times longer than that of unbound C-peptide for control experiments where only RhCP is added, and W_D is the corresponding sum for the displacement experiments where a nonlabelled peptide or amino acid is added to displace RhCP.

RESULTS

Alanine substituted pentapeptides corresponding to the C-terminal part of human C-peptide. The autocorrelation function describing the fluorescence intensity variations for RhCP bound to human renal tubular cell membranes together with the calculated diffusion times and the corresponding weight factors (fractions) are shown in Fig. 1A. When 5 μM of the native human C-peptide, or its C-terminal pentapeptide segment (EGSLQ), was added to bound RhCP in postincubation, there was 80–90% displacement of RhCP, as reported previously (19). Postincubation with 5 μM **A**GSLQ did not result in detectable competitive displacement of bound RhCP (Fig. 1B). In contrast, postincubation of bound RhCP with 5 μM E**A**SLQ was accompanied by marked displacement (Fig. 1C), amounting to approximately 80%. Individual postincubations with the pentapeptides EG**A**LQ, EGS**A**Q, and EGSL**A** at 5 μM concentrations resulted in intermediate displacement values of bound RhCP, amounting to 28, 20, and 42%, respectively. An overview of the results is presented in Fig. 2 for all peptides tested.

Importance of Glu in competitive displacement of human C-peptide from renal tubular cells. A series of measurements was undertaken to examine the importance of Glu. Postincubation of bound RhCP with 5 μM Glu was found to elicit 50% displacement, while incubation with 5 μM Ala or GSLQ, i.e., the human C-peptide C-terminal tetrapeptide, failed to result in competitive displacement (Fig. 2).

Competitive displacement of human C-peptide by C-peptide fragments from human and rat, and by porcine and rat C-peptide. Human C-peptide(1–26) does not displace the full-length peptide (Fig. 2). Likewise, postincubation experiments with either 5 μM EVARQ (corresponding to rat C-peptide C-terminal pentapeptide), rat full-length C-peptide, or 5 μM ELGGGPGAG (corresponding to human C-peptide positions 11–19) resulted in no displacement of bound RhCP. However, porcine C-peptide was found to displace human C-peptide to about 80% (Fig. 2).

DISCUSSION

Specific structural features appear to be important for the capacity of C-peptide to bind to cell membranes and to elicit biological effects, as is the case for other peptide hormones (24). FCS studies established that C-peptide at physiological, low nanomolar, concentrations binds to human renal tubular cell membranes,

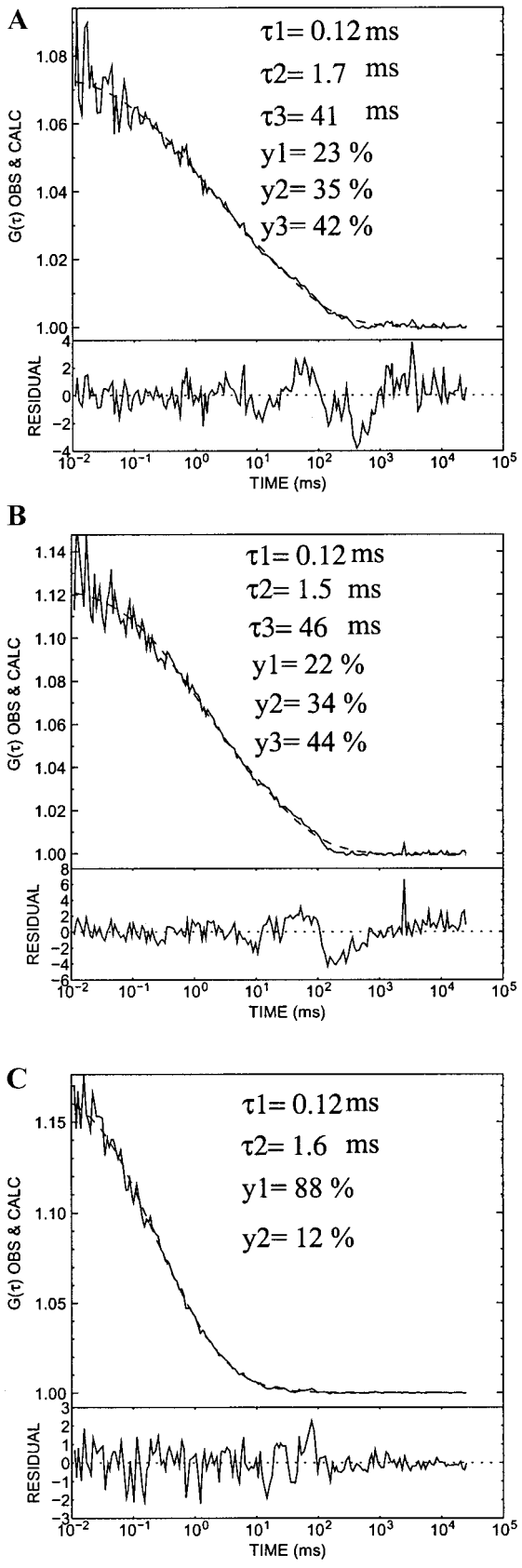

FIG. 1. C-peptide displacement with C-terminal pentapeptide. The fluorescence intensity autocorrelation function for RhCP bound to human renal tubular cell membranes after incubation with 5 nM RhCP for 60 min (A), for RhCP following postincubation with 5 μM **A**GSLQ for 3 h (B), and for RhCP following postincubation with 5 μM E**A**SLQ for 3 h (C). The observed diffusion times (τ) and weight factors (y) are denoted.

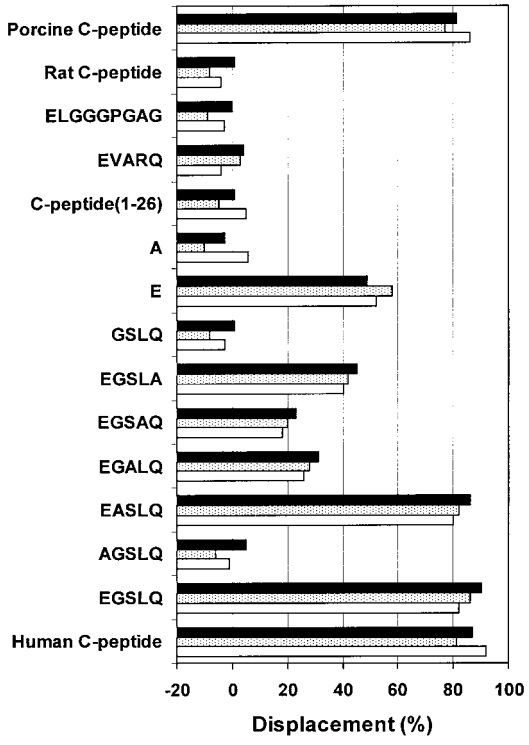

FIG. 2. Summary of competitive displacement of RhCP. The extent of displacement of 5 nM RhCP from human renal tubular cell membranes after postincubation with 5 μM of the following peptides and amino acids: human C-peptide, EGSLQ (human C-peptide C-terminal pentapeptide), **A**GSLQ, E**A**SLQ, EG**A**LQ, EGS**A**Q, EGSL**A**, GSLQ, E, A, C-peptide(1–26), EVARQ, ELGGGPGAG, rat C-peptide, and porcine C-peptide. For each peptide/amino acid the results of three independent experiments, each of which are the average of at least six measurements, are shown.

and that bound C-peptide is displaced by the C-terminal pentapeptide segment (19). These binding characteristics agree with results from studies of rat C-peptide effects on Na^+,K^+-ATPase activity in rat renal tubular cells (22), which show equal potency of C-peptide and its C-terminal pentapeptide segment. These results prompted us to examine the displacement capacity of the C-terminal pentapeptide segment. For this purpose we here chose to study binding of C-peptide in a quantitative manner using FCS. FCS measures diffusion velocities of fluorophore-labelled molecules across a minute laser focus. The diffusion time of a free ligand is shorter than that of a ligand associated with its receptor. It is therefore possible to determine whether a labelled ligand is free or bound; displacement of receptor-bound rhodamine-labelled

C-peptide is detected as a shift to the left of the autocorrelation function (19, 20). Using this technique with different ligands, the most intriguing result now obtained is that Glu27 is important in C-peptide binding (Figs. 1 and 2). Thus, C-peptide(1–26) or C-peptide(28–31), i.e., the tetrapeptide $G_{28}SLQ_{31}$, does not displace C-peptide, while C-peptide(27–31), i.e., the C-terminal pentapeptide $E_{27}GSLQ_{31}$ fully displaces the full-length peptide. Furthermore, Glu only, but not Ala, displace C-peptide, and an alanine scan shows that the Glu residue of the C-terminal pentapeptide segment, corresponding to Glu27 of the intact peptide, is essential for displacement (Fig. 2).

Peptide hormones often carry a limited number of functional groups that are essential for receptor binding (24). It would then seem as if C-peptide binding to cellular targets is mediated by its C-terminal pentapeptide segment, with Glu27 being crucial for this interaction. This view is supported by the finding that rat C-peptide(27–31), but not rat C-peptide(1–26) stimulates Na^+,K^+-ATPase activity in rat renal tubular segments (22). The possibility that glutamic acid as such, rather than the C-terminal pentapeptide segment is important for C-peptide binding and function is supported by two findings. Firstly, free glutamic acid displaces C-peptide to about 50%, while $G_{28}SLQ_{31}$ completely lacks potency in this respect. If the entire C-terminal pentapeptide segment mediates binding, partial displacement by GSLQ would be expected, in particular as the four "Ala scan" peptides, E**A**SLQ, EG**A**LQ, EGS**A**Q, and EGSL**A**, all partially displace C-peptide (Fig. 2). Secondly, the importance of Glu in C-peptide binding is indicated by results which show that peptides having a terminal Glu residue, including C-peptide, are possible effectors for binding to *N*-methyl-D-aspartate (NMDA) subtypes of glutamate receptors (25). Notably, all four Glu residues of C-peptide, together with three Gly, two Gln and two Leu residues are the only residues strictly conserved in human, pig, rat and bovine C-peptide. The unique importance of Glu, however, is contradicted by the inability of the Glu-containing rat C-peptide C-terminal pentapeptide, EVARQ, and human C-peptide positions 11–19 (ELGGGPGAG), to displace human C-peptide (Fig. 2). Moreover, the C-terminal tetrapeptide of rat C-peptide, VARQ, stimulates Na^+,K^+-ATPase activity in rat renal tubular segments almost to the same extent as the full-length peptide or the C-terminal pentapeptide does (21), also suggesting that not only Glu dictates C-peptide action.

An internal segment of C-peptide (encompassing at least residues 11–19) has been shown to stimulate Na^+,K^+-ATPase activity (22) and to influence vascular function in rats with experimentally induced diabetes (16). Surprisingly, this internal peptide (ELGGGPGAG) has no capacity to displace C-peptide from human renal tubular cells (Fig. 2). This suggests that C-peptide, and fragments thereof, may elicit biological effects via more than one pathway, or works differently between rat and man. This supports the notion that several mechanisms, also nonstereospecific interactions may to be involved in mediating C-peptide effects, cf. introduction.

In summary, we show that Glu27 of C-peptide is particularly important for specific binding to human renal tubular cells, and that although Glu competes with C-peptide binding, not all Glu-containing peptides do so. Different receptors for one ligand have been shown before, also regarding the proinsulin family of ligands, e.g., for IGF-1 (26). The possibility should be considered that C-peptide binding and function may be mediated via more than one receptor, explaining why the concept of C-peptide as specific ligand has been difficult to establish.

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