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Research Article

Cellular internalization of proinsulin C-peptide

E. Lindahl[a], U. Nyman[b], E. Melles[a,+], K. Sigmundsson[c,++], M. Ståhlberg[a], J. Wahren[d], B. Öbrink[c], J. Shafqat[a], B. Joseph[b] and H. Jörnvall[a,*]

[a] Department of Medical Biochemistry and Biophysics (MBB), Karolinska Institutet, 171 77 Stockholm (Sweden), Fax: +46-8-337462, e-mail: Hans.Jornvall@mbb.ki.se
[b] The Institute of Environmental Medicine (IMM), Karolinska Institutet, 171 77 Stockholm (Sweden)
[c] Department of Cell and Molecular Biology (CMB), Karolinska Institutet, 171 77 Stockholm (Sweden)
[d] Department of Molecular Medicine and Surgery (MMK), Karolinska Institutet, 171 77 Stockholm (Sweden)

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Abstract. Proinsulin C-peptide is known to bind specifically to cell membranes and to exert intracellular effects, but whether it is internalized in target cells is unknown. In this study, using confocal microscopy and immunostained or rhodamine-labeled peptide, we show that C-peptide is internalized and localized to the cytosol of Swiss 3T3 and HEK-293 cells. In addition, transport into nuclei was found using the labeled peptide. The internalization was followed at 37°C for up to 1 h, and was reduced at 4°C and after preincubation with pertussis toxin. Hence, it is concluded to occur via an energy-dependent, pertussis toxin-sensitive mechanism and without detectable degradation within the experimental time course. Surface plasmon resonance measurements demonstrated binding of HEK-293 cell extract components to C-peptide, and subsequent elution of bound material revealed the components to be intracellular proteins. The identification of C-peptide cellular internalization, intracellular binding proteins, absence of rapid subsequent C-peptide degradation and apparent nuclear internalization support a maintained activity similar to that of an intracrine peptide hormone. Hence, the data suggest the possibility of one further C-peptide site of action.

Keywords. Proinsulin C-peptide, cellular internalization, confocal microscopy, immunostaining, rhodamine-labeling, surface plasmon resonance, peptide mass mapping.

Introduction

In proinsulin, C-peptide constitutes a connecting segment between the B and A chains of insulin. After processing, it is released with insulin from the pancreatic β-cells [1]. C-peptide has long been recognized to promote the folding of proinsulin, but it is now also recognized to be a bioactive peptide as evidenced by cellular and signaling effects, effects in animal experiments, in clinical studies of patients with type 1 diabetes [2–4], and most recently, in disaggregation of insulin oligomers [5]. C-peptide binds specifically to cell membranes from pancreatic β-cells, human renal tubular cells, human fibroblasts and endothelial cells [6–9]. The observations suggest that these cells may have membrane receptors for C-peptide, often suggested to be G-protein-coupled receptors (GPCRs) as deduced from effects of per-

[+] Present address: Development DMPK & Bioanalysis, AstraZeneca R&D Södertälje, 151 85 Södertälje (Sweden).
[++] Present address: Niels Bohr Institute, Copenhagen University, Blegdamsvej 17, 2100 Copenhagen, Denmark.
* Corresponding author.

tussis toxin (PTX). There are also indications that certain C-peptide effects may be mediated by non-chiral interactions rather than stereospecific receptors or binding sites [10]. However, these activities have been questioned [11], and fluorescence correlation spectroscopy suggests a specific interaction of C-peptide with cell membranes as well as nonspecific interactions of a minor component [7]. Hence, further studies on C-peptide cellular interactions are necessary.

Localization of peptides within cells and interactions with proteins or other peptides can be studied with immunofluorescence, which has the advantage of a label-free system, allowing for retrieval of peptide localization through specific monoclonal antibodies. We now turned to this method to investigate whether C-peptide is internalized and, if so, the subcellular location of C-peptide. In addition, surface plasmon resonance (SPR) was used to study the binding of cell extracts to C-peptide. We present evidence that C-peptide is transported across cell membranes and in accordance with this observation, we find binding of intracellular proteins to C-peptide.

Materials and methods

Cell culture. HEK-293 and Swiss 3T3 cells were maintained in Dulbecco's modified medium (D-MEM) (Gibco) containing 10% (v/v) fetal bovine serum (FBS) (Gibco) and 25 μg/mL gentamycin (Gibco) at 37°C in humidified air with 5% CO_2. Cells at 50–70% confluency were used in all experiments. Serum-starved cells were maintained overnight in D-MEM, supplemented with 25 μg/mL gentamycin.

C-peptide localization and confocal microscopy imaging. HEK-293 and Swiss 3T3 cells were seeded on coverslips, allowed to settle and serum-starved overnight. Cell monolayers of HEK-293 and Swiss 3T3 cells were treated at 37°C with 10 μM C-peptide (Schwarz Pharma AG) dissolved in serum-free medium. In control wells, a corresponding amount of serum-free medium was added. After treatment, cell layers were washed with cold Hank's balanced salt solution (HBSS) (Gibco) or phosphate-buffered saline (PBS) (Gibco), prior to immunostaining. Cells on coverslips were fixed in 4% formaldehyde, blocked in 10 mM HEPES with 0.3% Triton X-100 and 3% bovine serum albumin (BSA), incubated with anti-human C-peptide mouse monoclonal antibodies (Medix Biochemica) overnight at 4°C and then with Alexa Fluor 488-conjugated goat anti-mouse antibodies (Molecular Probes) for 30 min at room temperature. Cell nuclei were counterstained with Hoechst 33342 (1 μg/mL). Samples were mounted in Vectashield H-1000 mounting medium (Vector Laboratories, Inc.) and analyzed in a Zeiss 510 Meta confocal laser-scanning microscope, equipped with an inverted Zeiss Axiovert 200M. Mixed dyes were acquired by sequential multiple channel fluorescence scanning to avoid bleed through.

Flow cytometric analysis. Swiss 3T3 cell uptake of peptide was determined by flow cytometric analysis using a FACSCalibur flow cytometer equipped with CellQuest software (Becton Dickinson). Cells were treated as described above, harvested and fixed in 1% formaldehyde, blocked in 10 mM HEPES with 0.3% Triton X-100 and 3% BSA, and incubated with primary antibodies overnight at 4°C and then with Alexa Fluor 488-conjugated goat anti-mouse antibodies for 1 h at room temperature. As a control that primary antibody was specific for an intracellular target, fixed samples were blocked under non-permeabilizing conditions in 10 mM HEPES with 3% BSA, and then subjected to primary and secondary antibodies. Samples were analyzed using the BD CellQuest software and a primary gate based on physical parameters (forward and side light scatter, FSC and SSC, respectively) was set to exclude dead cells and debris. In each sample, 10000 cells were analyzed based on the intracellular presence of fluorescent secondary antibody.

Preparation of labeled C-peptide. Rhodamine-labeled human C-peptide was prepared with a tenfold molar excess of tetramethyl rhodamine (Rh) (absorption 555 nm, emission 580 nm), using a succinimidyl ester derivative (Fluoreporter F-6163, Molecular Probes). Labeled C-peptide was separated from excess dye and unlabeled material by reverse phase high-performance liquid chromatography (HPLC) on a C18 column (4.6×250 mm, particle size 5 μm) (Grace Vydac). Elution was performed at 1 mL/min with a gradient of 0–60% acetonitrile in 0.1% trifluoroacetic acid (TFA). The absorbance was monitored at 214 and 280 nm. Fractions containing Rh-labeled C-peptide were identified by matrix-assisted laser desorption ionization (MALDI) mass spectrometry (MS) with an Applied Biosystems Voyager DE-PRO instrument, pooled and lyophilized. The HPLC step was found to result in complete separation of labeled and unlabeled peptide, thus producing Rh-labeled C-peptide essentially pure. Concentration of labeled C-peptide was determined by amino acid analysis in a Biochrome 20 plus instrument (Bie & Berntsen) after hydrolysis with 6 M HCl/0.5% phenol in evacuated tubes at 110°C. Biotin-labeled peptides were prepared according to manufacturer protocols (Biotin labeling kit, Roche). In brief, C-peptide or scrambled C-peptide was dissolved in PBS and a tenfold molar excess of D-biotinoyl-ε-aminocaproic acid-*N*-hydroxysuccinimide ester was added. The solution was kept at room temperature for 2 h under gentle stirring. Reaction mixtures were then desalted on PD10 columns (Pharmacia). Separation of labeled peptides from unlabeled material was achieved by reverse phase HPLC on a C18 column (4.6×250 mm, particle size 5 μm) (LKB Ultropak TSK ODS-120T). Elution was performed at 1 mL/min with a gradient of 0–100% acetonitrile in 0.1% TFA. Fractions containing biotinylated peptides were identified by MALDI-MS. The HPLC step was found to result in complete separation of labeled and unlabeled peptide, thus producing biotin-labeled C-peptide essentially pure. Concentrations were determined by amino acid analysis as described above.

Rh-labeled C-peptide localization and confocal microscopy imaging. Cells were seeded on coverslips, allowed to settle and serum-starved overnight. Monolayers of Swiss 3T3 cells were stimulated at 37°C for 30 min with 1 μM Rh-labeled C-peptide dissolved in serum-free medium. In control wells, a corresponding amount of free Rh dissolved in serum-free medium was added. After treatment, cell layers were washed with cold PBS prior to fluorescence imaging. Cells on coverslips were fixed in 4% formaldehyde. Cell nuclei were counterstained with Hoechst 33342 (1 μg/mL), and cells were visualized as previously described.

Effects of temperature and PTX on Rh-labeled C-peptide localization. Monolayers of Swiss 3T3 cells prepared as described above, were treated at 4°C for 30 min with Rh-labeled C-peptide dissolved in serum-free medium. In control wells, a corresponding amount of free Rh dissolved in serum-free medium was added. Cells were also incubated with 200 μg/mL PTX (Sigma) for 6 h before treatment with Rh-labeled C-peptide and free Rh as control as previously described. After treatment, cell preparation and visualization was as described above.

Time-lapse confocal microscopy. Cells were grown on glass coverslips (42 mm diameter). The coverslips were assembled in an "open" cell cultivation system (PeCon GmbH) and cells were incubated with serum-free medium containing Hoechst 33342 (1 μg/mL) for 5 min at 37°C. Rh-labeled C-peptide was added and time-lapse image capturing was started a few seconds after peptide addition. The procedure was also performed with free Rh. The imaging of live cell was done at 5-min intervals for 50 min.

Immobilization of peptides on Biacore sensor chips. Streptavidin-

coated (SA) sensor chips (Biacore AB) were used for immobilization of biotinylated peptides. Briefly, 0.1 mg/mL of biotin-labeled peptides in Biacore running buffer containing 0.01 M Tris, pH 7.4, 3 mM EDTA, 0.005% surfactant P20, and 0.15 M NaCl (Tris-buffered saline, TBS) were applied to a SA-chip. C-peptide and scrambled C-peptide, respectively, were immobilized at different sensor surfaces on the same sensor chip. For regeneration of the sensor chip, 6 M guanidine-HCl, pH 6.8, was used.

Cellular extracts and SPR identification of binding proteins. HEK-293 cells grown on culture dishes were collected with a rubber policeman and centrifuged at 4°C for 4 min at 2000 *g*. Solubilization was carried out in cell to solute (TBS) ratio of 1:6 in the presence of 0.4% deoxycholate for 30 min at 4°C, with addition of a complete protease inhibitor cocktail (Roche) and endonuclease (Sigma). The cellular extracts were centrifuged at 100 000 *g* for 30 min, and the supernatant was used for SPR studies.

Using a Biacore 3000 instrument, the HEK-293 cellular extract was allowed to pass over biotinylated peptides immobilized to an SA-chip at 10 μL/min at 25°C. Recorded data were scale-transformed, background subtracted, evaluated, and plotted in the BIA evaluation program 3.1. Recoveries from chips were performed with 10–22 repeated cycles. In each cycle, the sample was first allowed to pass over the C-peptide-labeled surface, the surface was then washed with Biacore running buffer for 2 min, and at the same time a bypass wash with 2 M NaCl was performed to clean the flow channels. After the wash with Biacore running buffer, bound material was eluted with the Biacore microrecovery method. For this, 4 μL 1% TFA recovery solution was introduced in between two air bubbles [12, 13] and brought to the chip, where the solution was allowed to remain by switching off the pump for 2 min to disrupt the binding, after which the pump was restarted and the solution recovered in a tube. The bound fraction recovered from the HEK-293 cell extract was adjusted to pH 8 with 0.1 M NaOH, and further adjusted to 50 mM ammonium bicarbonate by addition of 1 M ammonium bicarbonate. Trypsin (1 μL of a 1 μg/μL solution in 50 mM acetic acid) was added, and incubations were carried out for 5 h at 37°C and stopped by freezing. Peptides generated were analyzed by MALDI-MS, and the masses screened for protein identification as described [14].

Results

Localization of C-peptide using immunostaining and confocal microscopy. To establish whether C-peptide is internalized, C-peptide was incubated with the mouse fibroblast cell line Swiss 3T3 and the human embryonic kidney cell line HEK-293. The localization of C-peptide in cells was visualized employing immunostaining and confocal laser-scanning microscopy (Fig. 1a, b). After 30 min incubation at 37°C with C-peptide (10 μM), an almost homogeneous distribution of the peptide was observed in the cytosol of both cell types. Analysis of immunostained samples not treated with C-peptide resulted in a low degree of background fluorescence as exemplified by the FACS experiment (Fig. 1d, green curve).

Flow cytometric analysis of C-peptide localization. The cellular localization of C-peptide was also investigated by flow cytometric analysis in both permeabilized and non-permeabilized Swiss 3T3 cells (Fig. 1), employing a similar protocol as for the immunostaining. Flow cytometry was used to verify that the primary antibody indeed had an intracellular rather than a membrane-bound target. Thus, after 30 min incubation at 37°C of C-peptide with Swiss 3T3 cells, the internalization could not be detected when cell permeabilization was omitted (Fig. 1c). No displacement in fluorescence intensity occurred without permeabilization since antibodies could then not reach the target, C-peptide. The same treatment with C-peptide followed by subsequent cell permeabilization resulted in a detectable intracellular localization of C-peptide (Fig. 1d).

Fluorescence imaging of Rh-labeled C-peptide. Rh-labeled C-peptide (1 μM) was incubated with Swiss 3T3 cells. Each peptide contained one Rh-moiety as determined by MALDI-MS. The intracellular distribution of C-peptide was visualized employing confocal laser-scanning microscopy directly detecting Rh-labeled C-peptide (Fig. 2). After 30-min incubation at 37°C, a homogeneous distribution of Rh-labeled C-peptide was observed in the cytosol, with staining also in nuclei (Fig. 2b, c). Rh alone did not give any cytosolic or nuclear staining.

Effects of temperature and PTX on Rh-labeled C-peptide localization. Rh-labeled C-peptide was visualized employing confocal laser-scanning microscopy. The internalization of Rh-labeled C-peptide at 4°C was significantly reduced *versus* that at 37°C (Fig. 2d). Swiss 3T3 cells were preincubated for 6 h with the G-protein inhibitor PTX. After treatment, cells were incubated as previously described with Rh-labeled C-peptide and the intracellular distribution of C-peptide was visualized employing confocal laser-scanning microscopy. PTX significantly reduced the internalization of Rh-labeled C-peptide resulting in a similar absence of fluorescence as seen in Figure 2d.

On-line imaging of Rh-labeled C-peptide localization. Rh-labeled C-peptide uptake in Swiss 3T3 cells was monitored on-line using time-lapse confocal microscopy. Starting from addition of peptide, cells were monitored every 5 min for 50 min (Fig. 2). Internalization started after 10 min and then continued slowly, with a more pronounced internalization starting to occur after 20 min. It was observed that a few cells in the population could exhibit slower internalization rates, as observed in Figure 2e, where at 30 min not all cells contained Rh-labeled C-peptide. For a cell population containing little Rh-labeled C-peptide, the progress of internalization in one cell can be followed in the bottom charts of Figure 2. At 30 min, this cell contains no Rh-labeled C-peptide, as seen from the absence of fluorescence inside the cell borders. By 50 min, the cell contains Rh-labeled C-

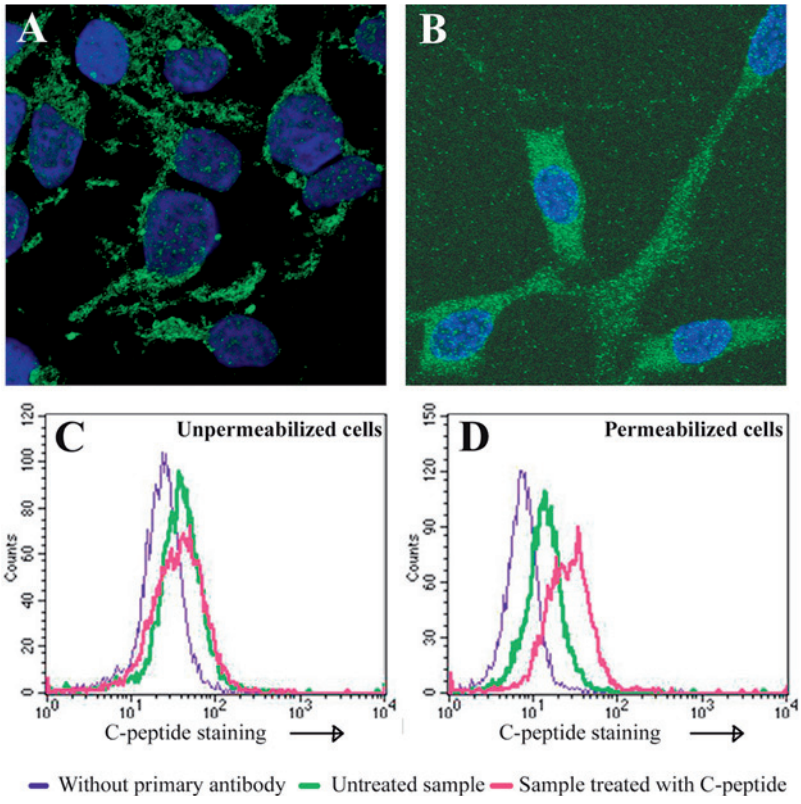

Figure 1. The intracellular distribution of immunolabeled C-peptide examined with confocal microscopy and FACS. Swiss 3T3 and HEK-293 cells were incubated with C-peptide (10 μM) for 30 min at 37°C and samples were prepared as described in Materials and methods. HEK-293 cells (*a*) and Swiss 3T3 cells (*b*), treated with C-peptide and immunostained viewed with confocal microscopy. Analysis of samples not treated with C-peptide resulted in low background fluorescence as exemplified by the FACS experiment (*d*, green curve). Swiss 3T3 cells; control, untreated and treated with C-peptide, immunostained unpermeabilized (*c*) or permeabilized (*d*) and analyzed with FACS. Blue curve, secondary antibody control sample; green curve, untreated sample; pink curve, treated sample. Each figure shown represents three individual experiments.

peptide, as seen by the presence of fluorescence within the cell borders. The intracellular distribution of Rh-labeled C-peptide was observed in both cytosol and nuclei.

Binding of HEK-293 cell extracts to C-peptide. The binding of HEK-293 cell extract components to immobilized biotinylated C-peptide was studied. As a control, biotinylated scrambled C-peptide (same amino acid composition but random sequence) was used. In tests with differently solubilized homogenates, it was established that 0.4% deoxycholate as detergent produced the best measurements of binding to immobilized C-peptide (Fig. 3). When HEK-293 cell extracts were applied to chips already exposed to C-peptide antibodies, no further binding was detected, showing that the HEK-293 extract binding is specific for C-peptide. Furthermore, in competition experiments where cellular extracts were pre-incubated with 10–100 nM C-peptide, a concentration-dependent decrease in the binding to C-peptide sensor surfaces was found (data not shown), again demonstrating specificity in the binding of HEK-293 cell extract molecules to C-peptide. Addition of GTPγS and Mg^{2+} to the cell extracts was tested since these compounds influence ligand binding to GPCRs, and several studies have suggested involvement of a GPCR component in the C-peptide binding to kidney cells [7, 15]. Using the HEK-293 cell extract, the addition of 50–100 μM GTPγS during extraction gave a concentration-dependent decrease in the specific binding to C-peptide, and 10 mM Mg^{2+} in the extraction buffer stabilized the binding as noticed by measurements with and without the Mg^{2+} preincubations (data not shown).

Identification of binding proteins. Having established specific binding of HEK-293 cell extract components to C-peptide, binding proteins were eluted using the Biacore microrecovery method and identified by peptide mass fingerprinting. Recovery of bound material from elution with 1% TFA for the HEK-293 cells resulted in removal of about 90% of the bound sample. Complete regeneration of the surface prior to the next experiment was possible with 6 M guanidine-HCl, pH 6.8. The eluted material was digested with trypsin and analyzed by MS. Although the Biacore chip gives only a single-step purification, this was sufficient to remove much protein, and to leave appreciable amounts of only a few intracellular proteins in the eluate. They were determined to be the α-chain of spectrin (Q13813), myosin light chain kinase (Q15746), cytoskeletal keratin type II (1346343) and calcium/calmodulin-dependent serine protein kinase (CASK) (8101954). Accession numbers given represent Swiss-Prot or NCBI entries.

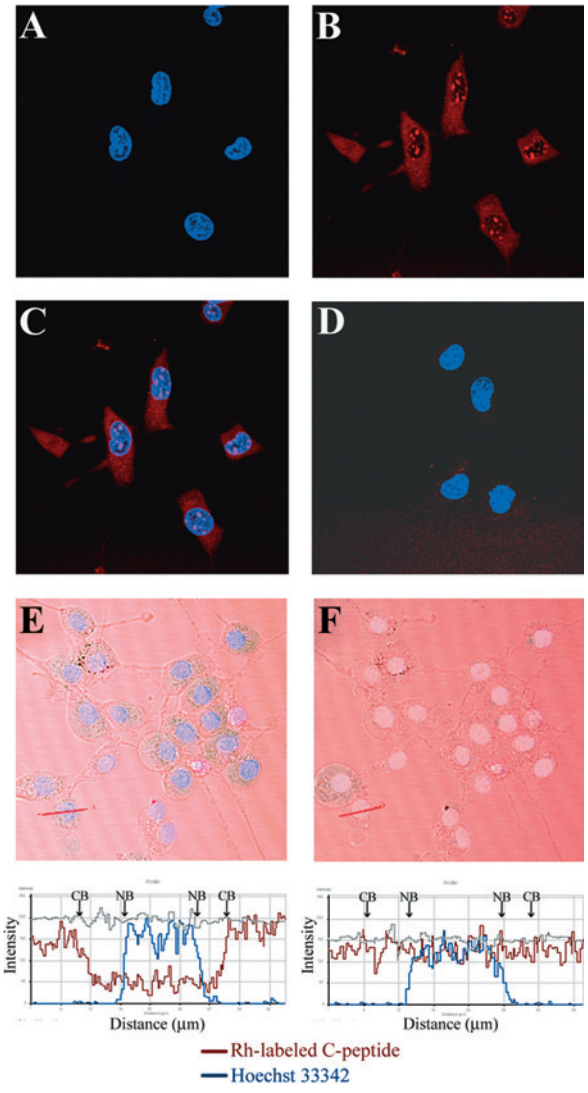

Figure 2. The intracellular distribution of Rh-labeled C-peptide examined with confocal microscopy (*a–d*) and on-line imaging confocal microscopy (*e*, *f*). Swiss 3T3 cells were incubated with Rh-labeled C-peptide (1 μM) for 30 min at 37°C (*a–c*) and at 4°C (*d*), or directly visualized at different time points after addition of peptide (*e*, *f*), as described in Materials and methods. (*a*) Nuclear staining with Hoechst 33342 in cells treated with Rh-labeled C-peptide; (*b*) Rh-labeled C-peptide staining; (*c*) overlay of *a* and *b*; and *d*, the effect of temperature (4°C). Treatment with Rh alone did not give any cytosolic or nuclear staining. Each figure shown represents three individual experiments. (*e*) Sample 30 min after Rh-labeled C-peptide addition (1 μM); and (*f*) sample after 50 min. Graphs below each frame correspond to fluorescence intensity in the cell crossed with the red line in (*e*) and (*f*). CB: Cell borders; NB: nuclear borders. At 30 min, the cell contains no Rh-labeled C-peptide as seen from the absence of fluorescence inside cell borders. At 50 min, the cell contains Rh-labeled C-peptide as seen by the presence of fluorescence within cell borders. Gray, phase contrast; blue, nuclear staining with Hoechst 33342; red, Rh-labeled C-peptide.

Spectrin α-chain, myosin light chain kinase [16] and cytoskeletal keratin type II represent cytoskeletal and cytoskeletal-associated proteins with spectrin α-chain also associated with the inner surface of the plasma membrane. CASK is a PDZ domain protein [17] localized to the cytosol and nuclei [18].

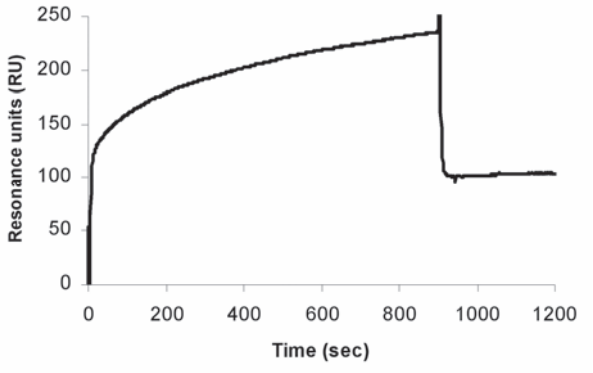

Figure 3. Differential binding of proteins from human HEK-293 cell extracts in 0.4% deoxycholate to C-peptide in SPR studies. The background-subtracted curve shows the binding to C-peptide

Discussion

Human proinsulin C-peptide has long been reported to exert bioactivity in cell lines, biochemical preparations, animals and diabetic type 1 patients [2, 3], and recently, a physiological role in insulin solubilization from pancreatic granulae was suggested [5]. Several studies show evidence of specific binding of C-peptide to cell membranes from primary cultures of pancreatic β-cells, human renal tubular cells, human fibroblasts and endothelial cells [6–9], compatible with the existence of a membrane receptor. The presence of a GPCR has been suspected from inhibition of effects by pretreatment with PTX [19–23]. However, the fate of C-peptide bound to cells is unknown. We now show that C-peptide is internalized in a mouse fibroblast cell line as well as in a human embryonic kidney cell line. In both cases, the internalization is energy-dependent and PTX sensitive. We further found that intracellular proteins bind to C-peptide as measured by SPR analysis.

Internalization of C-peptide was observed, with an almost homogeneous distribution of the peptide, in the cytosol after incubation at 37°C for 30 min (Figs 1 and 2). Two methods of detection were used, visualization of immunostained C-peptide and direct visualization of Rh-labeled C-peptide. These two methods were selected to accomplish both a sensitive (Rh-labeled C-peptide) and a label-free (immunostaining) system. Both methods gave a homogeneous cytosolic staining pattern of C-peptide, and FACS analysis confirmed the intracellular location of C-peptide. The concentration range (1–10 μM) used was well in line with other studies of peptide internalization [24, 25].

Although higher than physiological levels, the concentrations used are considered appropriate since effects of several peptide hormones *in vitro* often require considerably higher concentrations than applicable *in vivo* [26]. In the visualization of the Rh-labeled C-peptide, the staining was found to be localized to nuclei in punctuated spots. Rh alone was not detectable in nuclei, showing that the nuclear localization was not derived from the label. Also, it is not likely to be derived from label-induced alterations of the peptide, since other proteins are not affected by Rh-labeling [27]. The loss of C-peptide antigenicity in the nucleus is, therefore, deduced to reflect inadequate antigen accessibility to the antibody, as can occur in intracellular compartments [28, 29]. The advantageous sensitivity in visualization of Rh-labeled C-peptide allows detection, in spite of the antigen accessibility obstacles.

Using time-lapse confocal microscopy, the kinetics of C-peptide internalization was studied in greater detail. The internalization process began within 10 min of peptide addition and proceeded at a steady rate, as seen upon evaluation of uptake in individual cells, which was observed to proceed for 10–20 min. In the entire cell population, the internalization was observed to reach completion within 60 min.

To resolve whether the internalization is energy-dependent, and thus presumed to be endocytotic [30], or energy-independent, we studied internalization at 4°C. We found the internalization to be much decreased at this temperature, suggesting an energy-dependant process and implying that C-peptide is internalized via an endocytotic pathway. However, the classical features associated with endocytosis, such as punctuated staining in the cytosol and subsequent degradation, were not directly observed. The mechanism involved is unlikely to give direct C-peptide translocation across the plasma membrane. The inhibitory effect of PTX is most often derived from ribosylation of the α-subunit of G-proteins and frequently in association with a GPCR [31], but PTX-sensitive processes acting via guanine nucleotide-binding proteins not directly coupled to GPCRs also exist [32].

The finding that C-peptide is internalized prompted SPR analysis of binding interactions with cellular proteins. We found that components in cellular extracts bind to C-peptide (Fig. 3). The binding was selective for C-peptide *versus* scrambled C-peptide, and was not derived from unspecific chip surface effects. Binding components were present in cells (HEK-293) known to be sensitive to C-peptide effects. The decreased binding in the presence of GTPγS support the PTX-sensitive internalization of C-peptide now described. Proteins identified after elution reveal that intracellular components bind to C-peptide. Notably, some may be insignificant and just illustrate "sticky" proteins. Nevertheless, the proteins identified are of interest in view of the observed C-peptide internalization. CASK is a PDZ domain protein [17] predominantly localized to the cytosol, but also to membranes and nuclei [18]. Notably, PDZ domain proteins constitute a large family of proteins with the capacity to bind peptide ligands [33] with GPCR association [34]. Therefore, it is not inconceivable that binding C-peptide could be an intrinsic property of PDZ or PDZ-related proteins, and that this in turn could influence GPCRs, contributing to multiple or complex C-peptide effects in cells and tissues. The other proteins identified are cytoskeletal and cytoskeletal-associated proteins. The relevance of the proteins identified needs additional confirmation. Nonetheless, the proteins now identified with only a single-step purification are interesting in view of the C-peptide internalization.

There is an increasing body of evidence suggesting a new role for peptide hormones as intracrine factors, denoting intracellular hormones giving rise to cellular effects within the cell of synthesis or a target cell [35]. One prominent example is angiotensin, a secreted peptide known to pass from circulation to cardiac myocyte mitochondria and nuclei, where it has an effect on the transcription of platelet-derived growth factor (PDGF) with a maintained intracellular biological activity [36]. Other internalized intracrine peptides, including the fibroblast growth factor, are also reported to maintain an intracytoplasmic biological activity, indicating that peptides can have multifunctional activities, retaining a biological activity upon internalization, and with intracytoplasmic activities enhancing their biological multiplicity. Several reports show that C-peptide has effects at a transcriptional level, including for the PDGF-β receptor [37], anti-apoptotic B cell lymphoma 2 (Bcl2) protein [38] and a PTX-sensitive effect on the expression of vasopressin-activated calcium-mobilizing receptor [21]. That report, together with the present findings and a report of C-peptide activation of the transcriptional activity of peroxisome proliferator-activated receptor γ (PPARγ) in a PTX-sensitive fashion [19], raise the possibility that C-peptide is a true intracrine peptide hormone.

The recognition of C-peptide as a peptide capable of being internalized adds further information to the now large body of evidence on functions of C-peptide. The data expand the view that C-peptide is bioactive with now at least three distinct sites of function. It can apparently act directly on insulin oligomers without cellular mediators [5], on cellular surfaces via specific binding and subsequent signaling pathways [4, 7], and

on intracellular components via internalization (this study). Upon internalization, the pattern of C-peptide staining in the cytosol, as well as the additional nuclear localization of Rh-labeled C-peptide, suggests that C-peptide is not immediately degraded, indicating a maintained intracytoplasmic biological activity. This view is compatible with the identification of intracellular proteins obtained by C-peptide affinity chromatography.

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