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INTRACELLULAR AND PEPTIDE INTERACTIONS OF C-PEPTIDE

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One aspect of serendipity to bear in mind is that you have to be looking for something in order to find something else.

Lawrence Block

ABSTRACT

Proinsulin C-peptide is depleted together with insulin in type I diabetic patients. The supplement of insulin to these patients is necessary for their survival, but it is also likely that the loss of C-peptide may contribute to both short- and long-term complications. In this thesis, molecular effects of C-peptide have been investigated. In particular, nuclear effects of C-peptide and C-peptide involved in oligomerization have been studied, as well as method development to facilitate further protein interaction analysis.

C-peptide is a small peptide hormone with known membrane-binding properties that is thought to stimulate G-protein coupled receptor associated pathways. In this thesis we report that C-peptide not only acts extracellularly, but that it is internalized via specific mechanims and interacts with cytoskeletal proteins. We show that C-peptide is transferred to the nucleus, and specifically to the rRNA-synthesizing organelle nucleolus where it stimulates transcription of rDNA. Transcription of rDNA is related to a complex of proteins at the promoter region including histone 4 that gets acetylated upon interaction with C-peptide. We further link this transcriptional activity of C-peptide to proliferation in a model system relevant to the bone growth retardation observed in type I diabetic patients suffering from fractures. To understand more of C-peptide's effects on the basis of the transcriptional activity observed, a genome analysis of proximal tubular cells isolated from type I diabetic rats was performed. It revealed that C-peptide within 2 hrs exerts tight effects on transcription with ~500 genes affected and the majority of them being repressed. This observation suggests that C-peptide treatment corrects malfunctioning pathways, especially pathways of circulatory and inflammatory diseases.We have also studied oligomerization of C-peptide, and find that C-peptide oligomers are disrupted by insulin in addition to a previous study reporting that C-peptide disrupts insulin hexamers. The C-peptide oligomers are formed via electrostatic interactions, and can further lead to aggregates with a high content of ß-sheets.

In summary, this thesis provides data on C-peptide being an intracrine hormone with intracellular effects in addition to having extracellular activity via classical endocrinological pathways. We also discuss the implications of the C-peptide oligomers we observe, which provide evidence that C-peptide may act as an insulin chaperone. It is evident that a fine-balanced homestasis of C-peptide is necessary for optimal health in both type I and II diabetic patients.

LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to by their Roman numerals:

I: **Lindahl E**, Nyman U, Melles E, Sigmundsson K, Ståhlberg M, Wahren J, Öbrink B, Shafqat J, Joseph B, Jörnvall H. (2007) Cellular internalization of proinsulin C-peptide. Cell Mol Life Sci 64(4):479-86.

II: **Lindahl E**, Nyman U, Zaman F, Palmberg C, Cascante A, Shafqat J, Takigawa M, Sävendahl L, Jörnvall H, Joseph B. (2010) Proinsulin C-peptide regulates ribosomal RNA expression. J Biol Chem 285(5):3462-9.

III: **Lindahl E**, Nordquist L, Müller P, Agha E, Friederich M, Dahlman-Wright K, Palm F, Jörnvall H. Early transcriptional regulation by C-peptide in freshly isolated rat proximal tubular cells. *Submitted.*

IV: Jägerbrink T, **Lindahl E**, Shafqat J, Jörnvall H. (2009) Proinsulin C-peptide interaction with protein tyrosine phosphatase 1B demonstrated with a labeling reaction. Biochem Biophys Res Commun 387(1):31-5.

V: Jörnvall H, **Lindahl E**, Astorga-Wells J, Lind J, Holmlund A, Melles E, Alvelius G, Nerelius C, Mäler L, Johansson J. (2010) Oligomerization and insulin interactions of proinsulin C-peptide: Threefold relationships to properties of insulin. Biochem Biophys Res Commun 391(3):1561-6.

VI: Lind J, **Lindahl E**, Perálvarez-Marín A, Holmlund A, Jörnvall H, Mäler L. (2010) Structural features of proinsulin C-peptide oligomeric and amyloid states. FEBS J 277(18):3759-3768.

LIST OF ABBREVIATIONS

One letter codes for the 20 naturally occuring amino acids.

A	Alanine
R	Arginine
N	Asparagine
D	Aspartic acid
C	Cysteine
E	Glutamic acid
Q	Glutamine
G	Glycine
H	Histidine
I	Isoleucine
L	Leucine
K	Lysine
M	Methionine
F	Phenylalanine
P	Proline
S	Serine
T	Threonine
W	Tryptophan
Y	Tyrosine
V	Valine

acH4K16	Acetylated histone 4 lysine 16
APPL	Adaptor protein containing PH domain, PTB domain and leucine zipper motif
ATF	Activating transcription factor
ATR	Attenuated total reflection
bFGF	Basic fibroblast growth factor
CaMK	Calcium/calmodulin-dependent kinase
cAMP	Cyclic adenosine monophosphate
CASK	Calcium/calmodulin-dependent serine protein kinase
CD	Circular dichroism
ChIP	Chromatin immunoprecipitation
Cp	C-peptide

CREB	cAMP response element-binding
DLS	Dynamic light scattering
EGF	Epidermal growth factor
eNOS	Endothelial nitric oxide synthase
ER	Endoplasmatic reticulum
ERK	Extracellular signal-regulated kinase
FACS	Fluorescence-activated cell sorting
Ft	Fourier transform
GGH	Glycine glycine histidine
GPCR	G-protein coupled receptor
I	Insulin
IP	Immunoprecipitation
IR	Infrared
Kd	Dissociation constant
LC	Liquid chromatography
MS	Mass spectrometry
NMR	Nuclear magnetic resonance
NF-κB	Nuclear factor κB
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
pI	Isoelectric point
PI	Proinsulin
PKC	Protein kinase C
PP2B	Protein phosphatase 2B
PPI	Preproinsulin
PTHrP	Parathyroid hormone-related protein
PTP1B	Protein tyrosine phosphatase 1B
PTX	Pertussis toxin
PVDF	Polyvinylidene fluoride
qPCR	real-time quantitative PCR
Rh	Rhodamine
SDS	Sodium dodecyl sulfate
STAT3	Signal transducer and activator of transcription 3
TFE	Trifluroethanol
VACM-1	Vasopressin-activated Ca^{2+}-mobilizing receptor

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CONTENTS

INTRODUCTION

A PERSONAL REFLECTION

My first real contact with peptides was with cell-penetrating ones at Stockholm University. Then, my second was, as I arrived to the Department of Medical Biochemistry and Biophysics at Karolinska Institutet, antimicrobial peptides. They too, penetrate cell membranes. Thus, I was strongly biased to believe that other peptides always behave in this penetrative way. I still remember writing one of my first emails to my professor, suggesting that we should look at C-peptide, if it did so too. I got an encouraging reply that it was good to be curious and think wide. Eventually a combination of ideas, methodology, previous observations and collaboration got us on our quest, and we went into the midpoint of the cell. That quest continued us on the way of C-peptide oligomers, as our need of looking at Western blots of intracellular C-peptide made us observe the funny bands it occupied. But that's going too far ahead, too early.

To me, research about C-peptide has been like A journey to the centre of the earth and Around the world in eighty days – traveling through different spaces, finding adventure and fun. It has never been straightforward, or plain, instead this very small molecule has continued to elude us, showing new facettes, demanding of us creative skills and thinking. The C-peptide has sure made me a believer in the complexity of nature, its variability, and proneness to evolution.

This thesis is devoted to studies of C-peptide, its function, properties and ultimately, behaviour. I am fortunate to have been on this endevour with such open-minded and creative people.

> **Thinking is more interesting than knowing, but less interesting than looking.**
>
> Goethe

BACKGROUND

C-PEPTIDE ORIGIN

C-peptide was first described by Steiner et al. in 1967 (*1, 2*) in their work on the biosynthesis of insulin. The precursor proinsulin was proposed to exist to either protect insulin during intracellular transport or to aid in the assembly of the native insulin molecule. The latter was quickly found to be true, as C-peptide is vital for the proper formation of insulin disulphide bonds and hence the folding of insulin (*3*). It was later evidenced that even a precursor of pro-insulin itself existed, a very short-lived preproinsulin molecule degraded prior to completion of preproinsulin synthesis (*4, 5*).

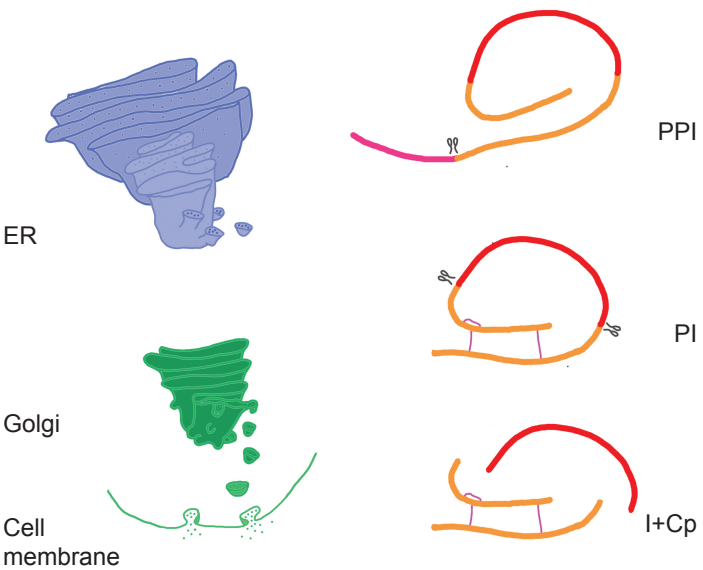

Figure 1. *Biosynthesis of C-peptide. To the left, a view of the cellular localization of biosynthesis is shown, and to the right, a scheme of the biosynthesis. Endoplasmatic reticulum (ER), preproinsulin (PPI), proinsulin (PI), insulin (I) and C-peptide (Cp).*

C-peptide is predominatly synthesized by ß-cells in the islets of Langerhans in the vertebrate pancreas, but evidence also exists of preproinsulin in the eye (*6*) and other parts of the brain (*7*), and in the gut (*8*). As shown in Figure

1, the precursor preproinsulin is directed to the endoplasmatic reticulum (ER) via its signal peptide that thereupon is cleaved off. Moving through the ER and the Golgi apparatus, the resulting proinsulin molecule is packed into secretory granules in which peptidases cleave proinsulin into insulin and C-peptide. These two peptides are then released into the blood stream in equimolar amounts upon stimuli.

C-peptide early provened to be valuable in clinical investigations of type I diabetes. Insulin is quickly depleted from the blood, and as C-peptide circulates for a longer time, it was recognized to be useful in immunoassaying residual insulin levels (*9, 10*). The following decade, most work on C-peptide focused on its role as a tool in the study of pancreatic disorders relying on its detectability in immunoassays. Also, basic investigations of its biological properties, including half-life (30 min) (*11*), major breakdown organ (kidney) (*12*) and sequence variability between species, were conducted. It was argued by some that C-peptide must lack any intrinsic biological function due to its sequence variability between species, although Kitabchi stated already in 1977 that ”the distribution of acidic, polar, and nonpolar amino acid residues along the C-peptide in proinsulin is similar from species to species” (*13*). The few investigations of C-peptide biological function that were carried out aimed primarily at finding insulin-like activity in insulin-sensitive tissues, and as no such effects were observed, C-peptide was generally held as a facilitator of insulin folding useful in tracing residual insulin secretion in diabetic patients.

C-PEPTIDE EFFECTS

Physiological responses to C-peptide

No story of C-peptide can be held separate from that of insulin, reported by Banting and Best in 1922 (*14*). Insulin was an enormous medical finding, saving thousands of lives annually, and the discovery of C-peptide was most probably associated with an anticipation of further understanding of type I diabetes. As Steiner et al. argue, the discovery of an insulin precursor could explain why type I diabetic patients have normal and even elevated levels of insulin at the disease onset, but it could also be a no-function vestige of an evolutionary ancestor (*1*). Further studies of C-peptide itself, aimed at finding

an activity similar to that of insulin, led C-peptide to be attached to its epithet "inactive byproduct" albeit several studies showed the opposite (*15*).

Studies of C-peptide effects on insulin secretion revealed that this co-secreted connecting peptide exerted negative effects on insulin release, suggesting a classical negative feedback inhibition mechanism (*16, 17*). Specific binding of C-peptide to insulin-secreting ß-cells was observed a few years later (*18*). Furthermore, hypoglycaemic activity of C-peptide (*17*) and stimulatory effects on glucose transport and glycogen synthesis were reported (*19*). The stimulatory effect on glucose utilization was observed also in type I diabetic patients together with a finding of C-peptide alleviating renal dysfunction (*20*). The involvement of C-peptide in glucose utilization and insulin activity came to dominate the following years apart from a study describing the C-peptide of a Locusta insulin-like peptide to have electrophysiological activity (*21*), acting on ion channel opening.

It is not inconceivable that the abundance of reports implicating C-peptide involvement in insulin activity and glucose utilization separate to that of the insulin receptor (*22*) led researchers to observe that diabetic patients with residual ß-cell function have improved blood glucose control, and even more, less suffering of retinopathy and microvascular lesions (*23, 24*). A continuos work on the benefits of C-peptide for type I diabetic patients, as summarized by Wahren et al. (*25*), ultimately discharged into research on the underlying protective molecular mechanisms of C-peptide.

Cellular effects

The inhibitory effect of C-peptide on insulin secretion and the binding to ß-cell membranes constitute two of the first observed cellular effects (*16, 18*). Specific binding to several human cell membranes was later observed and concluded to occur via a G-protein coupled receptor (GPCR) (*26*). As much effort focused on explaining observed physiological effects in the kidney, on glucose homeostasis and microcirculation, the abundant, ion channel Na^+K^+ATPase was investigated regarding its potential response to C-peptide. The ion channel was known to be targeted by many peptide hormones, and indeed C-peptide was shown to do so as well, possibly via calcium-activated protein phosphatase 2B (PP2B) and protein kinase C (PKC) (*27, 28*). C-peptide was also shown to increase calcium influx (*29*). All of these effects were inhibited by treatment with pertussis toxin, which is why a GPCR long

has been quested as a partner for C-peptide.

C-peptide has also been shown to have stimulatory effects on endothelial nitric oxide synthase (eNOS), connected to the beneficial effect of C-peptide on microcirculation (*30*). The effect on eNOS is mediated via extracellular signal-regulated kinase (ERK) pathway activation (*31*), hence also related to a GPCR activity.

Interactions of C-peptide

In the context of cellular activity, C-peptide has been shown to have specific binding to the cell membranes of ß-cells (*18*), renal tubular cells (*26*) and fibroblast cells (*32*). Rigler et al. showed the interaction to have a dissociation constant corresponding to a receptor binding, but they also showed a smaller fraction of nonspecific bindning (*32*). As no receptor has been identified, no detailed mechanism concerning the interaction recipient is known. Nonetheless, this receptor has been assumed to be a GPCR as several studies has shown that cellular effects of C-peptide are compromised by pertussis toxin (PTX) treatment (*27, 29*). However, PTX has been shown not to be as specific as presumed, and can instead be held as a disruptive agent of several cellular processes (*33*). Another study showed that a reverse sequence version of C-peptide and an enantiomer (D)-C-peptide version elicited the same biological activity as C-peptide, suggesting that the effect was not mediated via a receptor (*34*) but instead via non-chiral interactions. It was later shown that C-peptide can induce formation of cation (K^+)-selective channels in lipid bilayers (*35*), although other studies have shown no interaction between C-peptide and neutral or anionic vesicles and micelles (*36*).

Studies have shown that certain activities of C-peptide are connected to its metal-binding properties (*37, 38*), to zinc in particular. Zinc is an ion bioavailable to C-peptide in relevant amounts. In relevance to C-peptide's disaggregation of insulin hexamers (*39*), zinc can be released upon the disaggregation as the hexamers each contain two zinc atoms (*40*).

Properties of C-peptide

Human C-peptide consists of 31 amino acid residues as shown in Figure 2, has a molecular weight of 3020 Da, and an isoelectric point (pI) of 3. The peptide exhibits a large sequence variation between species, with glutamic residues 3 and 27 being the only residues highly conserved (*41*).However,

the N- and C-terminal portions of C-peptide, as well as the middle glycine-rich stretch are also relatively well conserved (*41, 42*), and as mentioned earlier, the distribution of certain groups of amino acids is similar (*13*).

Figure 2. *Amino acid sequence of C-peptide. Full and partial charges are shown as indicated. Amino acids are abbreviated as given in the List of abbreviations.*

C-peptide has been shown to have no ordered secondary structure as examined by circular dichroism (CD) spectroscopy, although trifluroethanol (TFE) can induce an α-helical structure in the N-terminal portion of C-peptide (*36*). This has been further supported by an extensive nuclear magnetic resonance (NMR) study, showing that the N- and C-terminal parts of C-peptide can adopt ordered structures (*42*).

INTRACRINE FACTORS

Chemical signaling has long been classified as endocrine, paracrine, juxtacrine and autocrine. In recent years, Re and colleagues have introduced the term intracrine which allows for an additional classification (*43, 44*), Figure 3. An intracrine peptide in their words describes a traditional endo-, para- or autocrine hormone that additionally is associated with an intracellular organelle not belonging to the secretory or degradatory pathway, ideally exerting a biological action on the basis of this association. This provides a new intracellular arena for understanding peptide hormones' and other signaling molecules' mode of communication. Previously, much signaling has been believed to occur via receptor binding.

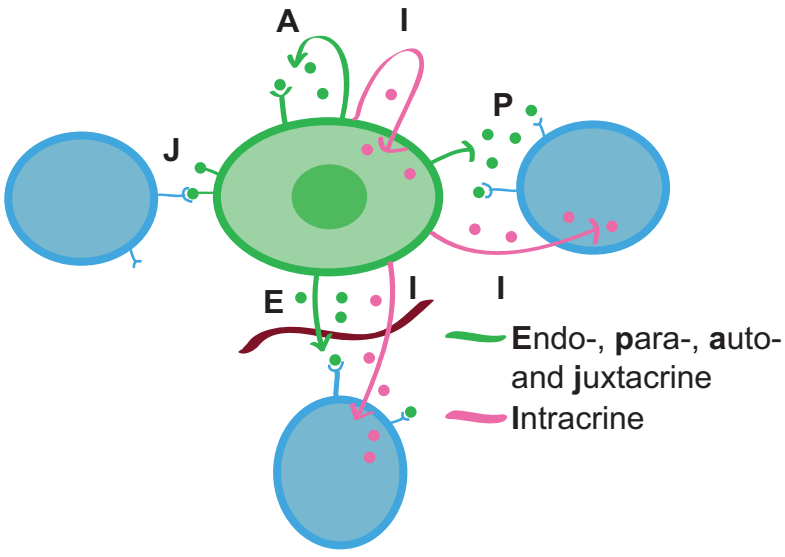

Figure 3. *Hormone signaling pathways. Classical endo-(E), para-(P), auto-(A) and juxtacrine(J) pathways (green) and intracrine(I) (pink).*

Intracrines share functional features; regulation of growth, effects on angiogenesis and interactions with the nucleus and nucleolus (*45*). It also seems as if intracrines modify the corresponding extracellular actions. Intracrine parathyroid hormone-related protein (PTHrP) has been shown to increase cell survival and growth, whereas extracellular PTHrP reduces both (*46*).

AIMS OF THIS THESIS

The overall aim of this thesis has been to understand cellular mechanisms by which C-peptide exerts its effects, and if possible, relate those findings to physiological responses of C-peptide.

More specifically, the work has been aimed at

- analysis of C-peptide interactions with the cell surface
- investigation of methods useful for protein interaction analysis
- investigation of C-peptide properties.

METHODOLOGY

Experimental procedures in **papers I-VI** are described below. Material and methods used in each study are described in **papers I-VI**.

BIOCHEMICAL METHODS

Enzymatic activity assay

For assay of protein tyrosine phosphatase (PTP) 1B activity in the presence of C-peptide and C-peptide fragments (**paper IV**), the enzyme and each peptide were preincubated to allow for binding to occur prior to addition of high-affinity and concentrated substrate. Activity of PTP-1B was determined by endpoint analysis.

Gel analysis

In this thesis, both polyacrylamide gel electrophoresis (PAGE) and agarose gel analysis have been performed (**papers II, IV and VI, and paper II**, respectively). PAGE has been used in both denaturing (**papers II and IV-VI**) and native (**papers V and VI**) conditions.

PAGE analysis

PAGE analysis is used to separate peptides and proteins according to charge and size (*47*). The analytical mixture is applied to a polymeric mesh of acrylamide, and separation is achieved based on the permeability of peptides and proteins as they are drawn through the mesh by an electrical field. With sodium dodecyl sulfate (SDS) PAGE, proteins are denatured and given a net negative charge by SDS, upon which they migrate based on size in an electrical field. In native PAGE, the undenatured proteins move according to their intrinsic charge and mass, and complexes and aggregates can be observed.

Agarose gel analysis

Agarose gel analysis is predominantly used for separation of nucleic acids, as it has a larger separation range than polyacrylamide.

C-peptide detection

Following PAGE, separated analytes can be visualized by staining methods (Coomassie and silver staining) or Western blot analysis. Coomassie and silver stains are widely used methods that rely on the analyzed peptide(s) or protein(s) to contain the functional groups required for staining. Silver stains interact predominaly with carboxylic acid, imidazole, sulfhydryl and amine groups (*48*). C-peptide, containing many carboxylic acid groups, should be expected to stain with silver. However, C-peptide only stains with Silver Stain Kit (Pierce), and only in very high amounts (>5μg). Coomassie dye stains with a chemistry primarily directed at lysine, histidine and arginine residues (*49*) – of which C-peptide contains none. Hence, C-peptide staining poses a problem. A C-peptide conjugate with rhodamine (Rh) can be used to directly visualize C-peptide in a gel (**paper V**).

Western blot allows for an alternative visualization as it is based on high affinity epitope recognigition by an antibody. There are several monoclonal antibodies for C-peptide that work well for immunofluorescence (**paper I**). Western blot analysis requires the analytes to adhere to either a polyvinylidene fluoride (PVDF) or a nitrocellulose surface. The adherence to a PVDF surface is based upon hydrophobic interactions, and to a nitrocellulose surface on a combination of hydrophobic and electrostatic interactions. C-peptide only in certain settings adheres to nitrocellulose membranes (**paper V**), and virtually never to a PVDF membrane. The use of biotinylated C-peptide circumvents the problem as a streptavidin-conjugate can then be used to visualize C-peptide, the biotin rendering better detection sensitivity and adherence properties (**papers V and VI**).

Mass spectrometry

Mass spectrometry (MS) has been used as described in **papers I, II, IV and V**. Tryptic digests have been analyzed by liquid chromatography (LC) tandem MS (MS/MS) (*50*) to identify C-peptide interacting proteins (**papers I and II**) and to establish an MS-compatible workflow (**paper IV**). In **paper V**, MS was used to analyze peptide interactions.

Protein interaction analysis

The interactions of C-peptide with other proteins and peptides have been studied in **papers I, II**, **IV and VI**. Techniques employed include the Biacore

technology, pull-down interaction analysis or affinity precipitation, co-immunoprecipitation, crosslabeling, native PAGE and MS. They are methodologically described in the separate publications.

C-peptide's negative charge (pI~3), small size (3 kDa) and lack of amino groups apart from its N-terminal one, pose problems when studying C-peptide interactions, as briefly discussed below.

One commonly used technique to capture interactions is immunoprecipitation (IP), used in **paper II** to capture histone 4 (chromatin IP (ChIP) analysis). This technique cannot be employed with C-peptide due to its small size, as it can become inaccessible to its antibody when interacting with proteins (compare cytosolic versus nuclear immunofluorescence intensity in **paper I**). To circumvent this problem we have used an N-terminally linked biotin-C-peptide. Biotin-streptavidin have a dissociation constant (Kd) in the order of ~10^{-15} mol/L, which makes it one of the strongest non-covalent interactions known. This phenomenon is utilized in the Dynabeads technology (Invitrogen) that allows for pull-down of biotinylated species by beads coated with streptavidin. Due to the previously mentioned properties of C-peptide, the biotin-C-peptide is first non-covalently attached to streptavidin beads, and then allowed to interact with an analytical mixture (**papers II and IV**). Biotin-C-peptide has also been used in Biacore experiments immobilizing C-peptide to streptavidin chips, as C-peptide due to its negative charge cannot be chemically attached to the commonly used Biacore carboxyl surface (**paper I**).

Crosslinking and crosslabeling are well used techniques as they allow for in vivo capture of protein interactions, as well as enrichment. Paraformaldehyde can be used to crosslink protein-protein and protein-DNA interactions with subsequent enrichment of interacting partners either via affinity tags or epitope recognition (as in ChIP analysis, **paper II**). Reactive groups can also be covalently attached to a bait, i.e. C-peptide, and then be activated to crosslink with interacting partners. However, this covalent attachment mostly relies on sulphydryl and amino group chemistry, and C-peptide contains only one amino group. Secondly, the bioconjugates are often of considerable size in relation to C-peptide.

A crosslabeling technique suitable to C-peptide's special prerequisites was adapted, described below (**paper IV**).

Crosslabeling

Brown et al. (*51, 52*) have described a crosslabeling method based on oxidation-mediated biotinylation of interacting species. A glycine-glycine-histidine (GGH) motif is introduced at the N-terminus, acting as a small histidine tag with high affinity for nickel. The nickel is oxidized and activates a biotin-conjugate that reacts within a defined space of the GGH-C-peptide, thereby biotinylating interacting species, Figure 4.

One important feature of this method is the sequestering of nickel. The GGH-tag only binds nickel above pH 6, and as C-peptide is acidic enough to significantly lower the pH of a solution, an ammonium acetate solution of pH 8.5 was used for nickel binding prior to crosslabeling. The use of ammonium actetate also facilitated the introduction of an MS-compatible step to allow for a workflow resulting in identification (**paper IV**). Following crosslabeling, biotinylated targets are enriched with the previously described Dynabeads technology, and either subjected to in-solution or in-gel tryptic digestion. Digests are then analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS).

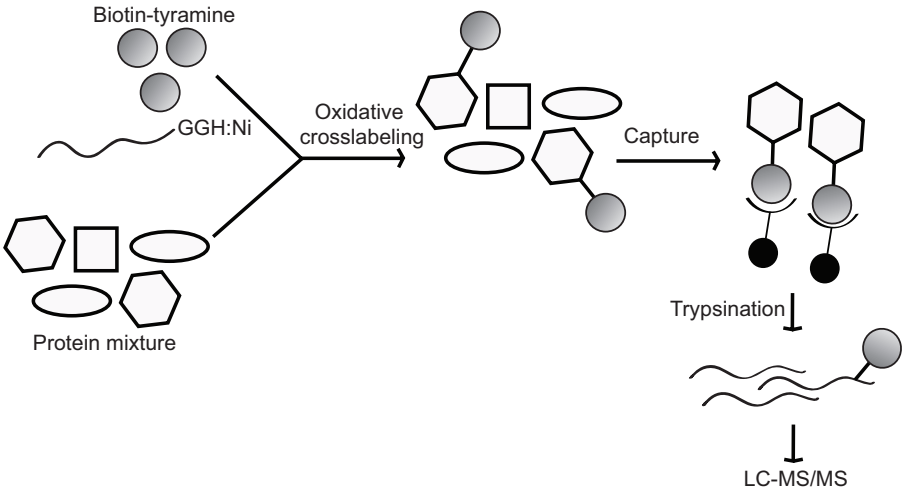

Figure 4. *Crosslabeling workflow. Incubation of bait peptide tagged with GGH:Ni and protein mixture with oxidative reagents mediates labeling of interacting species. Interaction partners are isolated and analyzed by mass spectrometry (LC-MS/MS).*

BIOPHYSICAL METHODS

Circular dichroism spectroscopy

CD analysis is used to analyze optically active molecules, including chromophoric proteins and peptides (*53*). How the chiral light interacts with a chiral sample, *i.e.*a peptide, gives information of the secondary structure. We used CD analysis in **paper VI** to monitor structure changes in different SDS concentrations.

Dynamic light scattering

Dynamic light scattering (DLS) is used to analyze the size distribution of suspended particles (*54*). In **paper VI**, DLS was used to analyze the size distribution of aggregates of C-peptide.

Infrared spectroscopy

Infrared (IR) spectroscopy gives details of molecular structure based on how the molecular bonds within a functional group can vibrate. In **paper VI**, attenuated total reflection (ATR) and Fourier transform (Ft) IR was used to study the secondary structure of oligomeric C-peptide.

NMR spectroscopy

The magnetic nuclear spin of certain atoms is a property that can be used to study structures, conformations and dynamics of molecules with NMR spectroscopy. In **paper VI**, diffusion NMR was used to study the size distribution of oligomeric C-peptide.

Thioflavine T staining

Thioflavine T is a dye that upon binding to aggregates of ß-sheet structure undergoes a characteristic transition in its emission spectrum (*55*).

CELL BIOLOGY METHODS

Cells and cell culture

In this thesis, HEK-293, Swiss-3T3, HCS-2/8 and proximal tubular cells have been used **(papers I and III-IV)**. HEK-293, or human embryonic kidney cells

from experiment nr 293, have been extensively used since the 70's (*56*) for their ease to handle. The cell type is suggested to resemble immature neurons (*57*). Swiss-3T3 cells originate from the 60's (*58*) and are derived from primary mouse embryonic fibroblast cells. Fibroblasts are commonly found in connective tissue. HCS-2/8 cells have a cartilage phenotype and are used as a model system for chondrocytes in human cartilage. They are derived from a human chondrosarcoma (*59*). Proximal tubular cells are found in the nephrons of the kidneys, and were isolated from diabetic rats (*60*).

Cells at 50–80% confluency in passage 6-20 were used in all experiments, with the exception of chondrocytes that were used between passage 3-7. For experiments with live cells, cells were seeded in cold medium and serum starved for a minimum of 12 hrs prior to the experiment. This was performed to synchronize cells and to avoid disturbance arising from growth factors, hormones or other serum components.
Proximal tubular cells were not grown but only maintained in culture for 2 hrs.

Cell viability analysis

The amount of cell death or proliferation reflects how a population of cells responds to external conditions. Analysis of viability was done as described in **paper II**.

DNA microarray

DNA microarray is a technique allowing for large-scale transcription analysis, utilizing the intrinsic capacity of an mRNA molecule to bind to its precursor DNA sequence. Multiple, short DNA sequences from a gene are fused to a chip, and the binding of a complementary mRNA molecule results in a readable answer. In **paper III**, DNA microarray was used to study the effect of C-peptide on transcription of genes in renal tubular cells isolated from diabetic rats.

Extraction of cellular material

For analysis of C-peptide and protein (**papers I and IV**), RNA (**paper II**) and histone interactions (**paper II**), protein extraction methods were employed as described in each paper. Usually, for binding analysis a large extraction from one passage set was performed in order to minimize passage-to-passage variation.

Gene reporter assay

To screen for the transcription of a gene in the absence or presence of different factors, it is valuable to do so in an intact cellular system. Reporter genes are constructs containing regulatory sequences of interest to the researcher, fused with a gene that upon expression results in a signal easy to identify and measure. These constructs are transfected into cells. In **paper II**, a luciferase construct was fused with an rDNA promoter sequence, and the emission of photons was monitored with a luminometer as a response to different experimental setups.

Fluorescence-activated cell sorting

Fluorescence-activated cell sorting (FACS) was used in **papers I and II**. The method relies on sorting cells based on their fluoresecent characteristics, as inferred to the cells by experimental procedures.

Immunofluorescence

The localization and abundance of molecules within a cell can be traced by their corresponding antibodies, and subsequently these antibodies are visualized with fluorescent conjugates. This technique was used in **papers I and II**.

Polymerase chain reaction analysis

Standard polymerase chain reaction (PCR) analysis has been used as described in **papers II and III**. For low-abundant genes, a relatively high number of cycles has been used.

STATISTICAL METHODS

Statistical analysis

Statistics deal with the numerical analysis of group- and population-sampled material, and serve to describe and infer properties of populations. In **paper II**, two-tailed paired student's t-test and one-way analysis of variance were used to compare differences between populations. In **paper III**, Mann-Whitney or Kruskal Wallis tests followed by Dunn's post hoc test were used to compare differences between populations.

RESULTS

PAPER I

Cellular internalization of proinsulin C-peptide

We investigated whether C-peptide is actively internalized into cells. Using confocal microscopy imaging of immunostained and Rh-labeled C-peptide we showed that C-peptide is internalized, and that it also enters into the nucleus. Combining protein interaction and identification analysis with the Biacore technology and mass spectrometry, we also found that C-peptide interacts with intracellular proteins, including cytoskeletal proteins and a multi-domain kinase protein.

PAPER II

Proinsulin C-peptide regulates ribosomal RNA expression

With the finding of C-peptide being localized to the nucleus in **paper I**, we also observed that C-peptide accumulates in nuclear subcompartments. Co-immunostaining revealed these compartments to be the rRNA-synthesizing nucleoli. C-peptide was found to rapidly induce transcription of rDNA, and to stimulate acetylation of and interact with histone 4 acetylated at lysine 16 (acH4K16) at the rDNA promoter region. As rRNA synthesis is tightly coupled to proliferation, we investigated and observed that C-peptide stimulates proliferation in chondrocytes and HEK-293 cells. Based on these findings we suggest C-peptide to have cell-specific growth factor activity.

PAPER III

Early transcriptional regulation by C-peptide in freshly isolated rat proximal tubular cells

The localization of C-peptide in nuclei and observed transcriptional effects showed in **paper II** made us want to investigate if there are any early-response genes that C-peptide regulates. In type I diabetic patients,

C-peptide supplement has been shown to improve renal function. We isolated proximal tubular cells from diabetic rats, incubated them with C-peptide for 2 hrs, and subsequently isolated mRNA that was analyzed with expression array genechips. ~500 genes were affected by C-peptide ($p<0.01$, fold change>1.3). Datamining of microarray data revealed that cardiovascular disease markers and diabetes complication markers are early reduced upon C-peptide stimulation.

PAPER IV

Proinsulin C-peptide interaction with protein tyrosine phosphatase 1B demonstrated with a labeling reaction

To find novel interaction partners of C-peptide and hence understand more of C-peptide biological function, we explored a robust method to study interactions of this small and acidic peptide. A nickel-catalyzed crosslabeling reaction employing a small tag was modified to suit our requisite of a mass spectrometry-compatible workflow. A previous study had reported C-peptide to influence phosphatase activity in intact cells (*61*), and with our method we could demonstrate that C-peptide interacts with PTP1B. Specifically, labeling data combined with enzyme activity analysis showed a functional interaction between acidic regions of C-peptide resembling PTP1B binding substrates and specific sites of PTP1B.

PAPER V

Oligomerization and insulin interactions of proinsulin C-peptide: Threefold relationships to properties of insulin

Insulin exists as monomers and hexamers in equilibrium. A previous study (*39*) has shown that C-peptide interacts with and disaggregates insulin oligomers, increasing insulin bioavailability as monomers, which elicit the physiological response. We show that this relationship is also reciprocal: C-peptide itself forms oligomers that are disaggregated by insulin. Thioflavine T staining shows C-peptide ß-sheet formation.

PAPER VI

Structural features of proinsulin C-peptide oligomeric and amyloid states

In **paper V**, we showed the presence of C-peptide oligomers. In this study we made an effort to characterize the formation process and oligomer features by trapping low-abundant oligomers with SDS. We show that the C-peptide oligomers are sensitive to divalent ions and NaCl, implicating electrostatic interactions involved in oligomer formation. We also show that the oligomers can form larger aggregates of ß-sheet structure, that SDS-induced oligomers are pH-sensitive, and that time is a contributing factor to oligomer formation.

DISCUSSION

In this thesis, findings are presented related to three topics; internalization of C-peptide and observations in connection to this (**papers I-III**), method development to allow for identification of C-peptide interacting partners (**paper IV**), and C-peptide oligomerization and properties thereof (**papers V-VI**). Below, the findings are discussed within these three sections followed by a more general discussion.

PAPERS I-III

Intracellular C-peptide

In **papers I-III**, the internalization of C-peptide and intracellular actions are described. In **paper I**, we show the internalization using two methods; confocal microscopy imaging of immunodetected and fluorescently labeled C-peptide (Rh-C-peptide), and FACS analysis. The methods evidence C-peptide as an intracellular species, and Rh-C-peptide was observed also in the nucleus. The internalization was later corroborated by another group that showed the internalization to occur via endocytosis of clathrin-coated vesicles (*62*). Both our and their later study show that internalization begins already within 10 minutes, although Luppi et al. describe the localization of C-peptide to be punctuate, whereas we describe it as homogenous. There is no real size-difference between the probes used in each study, so the discrepancy between localization pattern can most likely be explained by the use of different cell types or their observation of C-peptide in endosomes. Luppi et al. observe no nuclear localization.

In **paper I**, four proteins are identified as intracellular C-peptide binding components. Three are cytoskeletal proteins, whereas calcium/calmodulin-dependent serine protein kinase (CASK) is a multidomain scaffolding protein localized to the cytosol and nucleus (*63*). CASK contains a calcium/calmodulin-dependent kinase (CaMK) module (*64*) that is known to be involved in cellular responses induced by hormones and other signaling agents. CaMK targets include transcription factor cyclic adenosine monophosphate (cAMP) response element-binding (CREB) protein, implicated in C-peptide signaling

(*25*). CREB is functionally related to activating transcription factors (ATF) 3 and 4, that in **paper III** are shown to be affected by C-peptide. CASK also contributes to ion channel trafficking (*65*), which is interesting in the light of a study showing C-peptide ability to induce cation channels (*35*).

We suggested C-peptide to be an intracrine factor in **paper I**, and this report was recognized by others (*66*). How C-peptide and other intracrines traffick from membranes and endosomes to nuclear and other compartments is unclear. Several other intracrines, including epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF), have known membrane receptors with which they internalize, and release of peptide and receptor complexes in late endosomes can be mediated via the acidic milieu. Both EGF and bFGF appear in the nucleus, a pathway that for EGF requires internalization with its membrane receptor (*67*). EGF has also been found in a subpopulation of endosomes that upon EGF stimulation are decorated with adaptor protein containing PH domain, PTB domain and leucine zipper motif (APPL) proteins, followed by membrane release and nuclear localization of APPL proteins (*68*). Whether this mediates the nuclear localization of EGF or just models a signal relay is unclear. Nonenveloped viruses that accumulate in endosomes via clathrin-coated vesicles have been shown to escape endosomes via unknown membrane interactions (*69*). Also signal transducer and activator of transcription 3 (STAT3) and c-Met accumulate in endosomes prior to nuclear localization (*70*). The trafficking of c-Met to perinuclear compartments has been shown to be under the control of PKC, which is also a target of C-peptide (*28*). An interesting mechanism proposed by Cook et al., suggests that trafficking of endosomes through the late recycling pathway to perinuclear recycling compartments allows for retrograde movement of nuclear targets through subcellular membrane pathways (*71*).

The trafficking from membranes and endosomes to the nucleus poses a mechanistic problem that remains to be answered not only for C-peptide and other intracrines. Other hormones, including steroids, have to escape endocytotic compartments as well to reach their intracellular target upon endocytosis (*72*).

The specific C-peptide nuclear localization evidenced in **paper I** led us to the observation in **paper II** that C-peptide accumulates in the nucleolus and

stimulates rDNA transcription. Insulin has also been reported to stimulate rDNA transcription (*73*), although no nucleolar localization has been observed. Other intracrine factors such as bFGF and PTHrP also localize to the nucleolus, although PTHrP contains a nucleolar targeting sequence (*74*). The stimulation of rDNA transcription seems to be mediated via a complex situated at the 47S promoter. Interaction analysis revealed a binding between C-peptide and histone 4 (both non- and acetylated at K16). Analysis also showed an enrichment of acH4K16 at the promoter of 47S. C-peptide can induce acetylation of H4K16 within the time-frame of internalization, making it possible that C-peptide promotes this modification of H4K16 at the promoter. The acetylation of histone proteins is part of epigenetic mechanisms involved in chromatin remodeling, but it is unclear if C-peptide causes an epigenetic change or whether it is a time-limited modification, as we see a decrease in rDNA transcription already after 8 hrs.

C-peptide was also shown to interact with ribosomal protein S18. This is interesting not only because it provides an alternative verification of C-peptide nucleolar localization, but also because ribosome assembly occurs in the nucleolus (*75*). To expand speculation further, S18 provides an interesting link to both chemotaxis and CaMK-containing proteins such as CASK. C-peptide induces chemotaxis (*76*), and S18 interacts with cofilin (*77*), a protein involved in actin filament elongation (*78*). C-peptide has previously been shown to affect actin rearrangement (*79*) and in **paper I** binding to cytoskeletal proteins is observed. Although speculative, as reported in **paper II** and as evidenced in other studies, C-peptide does not physically interact with an abundance of proteins and those few that are interacting partners must be assumed to provide some insight into molecular function that can be pursued by experiments. S18 has also been shown to be a substrate for CaMK-proteins (*80*).

The increased transcription if 47S was further associated with proliferation in chondrocytes, a model system of interest in type I diabetes as ostopenia occurs in these patients. Type I diabetic patients also suffer from slow healing rates of fractures (*81*), and it has been suggested that apoptosis of chondrocytes is involved in the impaired bone health (*82*). C-peptide is shown in **paper II** to increase proliferation as well as to inhibit apoptosis in chondrocytes. The nuclear localization and transcriptional activation of the rDNA gene by

C-peptide observed in **paper II**, made us profile gene transcription activities of C-peptide. C-peptide has previously been shown to affect transcription of vasopressin-activated Ca^{2+}-mobilizing receptor (VACM-1) (*83, 84*) and eNOS (*31*), but as in the case of the VACM-1, long incubation times were used. The effect on eNOS expression was showed to occur via ERK pathway activation. Synergetic effects on transcription with other agents (*79*) and activation of the nuclear factor (NF) κB pathway via G-proteins (*85*) have also been observed.

Our goal was to catch early regulated genes, prior to subsequent activation of signaling cascades, and we therefore incubated proximal tubular cells isolated from type I diabetic rats with C-peptide for 2 hrs. This incubation time was chosen on the basis of the observed internalization rate in **paper I**, where C-peptide was first observed in the nucleus after 20-30 min with complete nuclear localization within 1 hr. We chose to study C-peptide effects in proximal tubular cells as one rapidly occurring effect of C-peptide in type I diabetic patients is normalization of glomerular hyperfiltration (*86*).

Analysis of mRNA from non-treated and treated samples with global gene expression profiling allowed us to observe which gene categories (*87*) that are predominantly activated. Categories related to transcriptional and GPCR activities were well represented, as were also catabolic enzymes, i.e. transferases. Of the ~500 genes affected by C-peptide, we chose ten candidates for real time quantitative (q) PCR analysis in order to validate the microarray analysis. The analysis confirmed activation of *Gpr87* and *Rup2* and repression of *Atf3*, whereas analysis of *Atf4*, *Tfec* and *Map3k8* gave inconclusive results. The increased transcription of *Ada*, *Mmp9*, *Tbx22* and *Foxo3a* opposed the results obtained using the Affymetrix platform. Several of the candidates chosen were low-expressed transcription factors, and only material from one control rat remained for the qPCR analysis.

The use of different probes and amplification techniques causes correlation problems when validating microarray results. Nevertheless, validation of candidates is important, in particular when identifying or pursuing a particular candidate of interest. In **paper III**, we focused on identifying overall trends and pathway association as this was the first microarray study to be conducted on C-peptide-stimulated samples.

The gene expression profiling allowed us to observe pathways that seem to be regulated by C-peptide, by employing different bioinformatic strategies

(*88, 89*). Most of the ~500 genes affected by C-peptide were repressed, suggesting normalization of diabetes-induced malexpressed genes. This was confirmed by pathway analysis that implied correction of several disease markers and mechanisms including cardiovascular disease markers and diabetes complication markers. No disease biomarkers or genes involved in disease molecular mechanisms were increased by C-peptide.

In **paper III**, 27,000 genes were analyzed and less than 500 genes were significantly regulated by C-peptide, indicating that C-peptide exerts a tight transcriptional regulation and confirming that the time-frame of 2 hrs was appropriately chosen.

PAPER IV

C-peptide crosslabeling

In any quest of molecular function, a bottom-up approach can be to identify interacting molecules and deduce function from this approach rather than going via function to mediators. There are many protein interaction techniques, including IP, crosslinking, two-hybrid systems and others. As dicussed in the methodology section, many of these protein interaction techniques are incompatible with the features of C-peptide. We tested a novel crosslabeling technique described in the literature by Brown et al. (*51, 52*), with compatible characteristics including a small tag. We used this technique to determine binding partners of C-peptide and found that our enzyme assay results showing C-peptide effects on PTP1B was in agreement with a physical interaction, even in more complex protein mixtures. We also used this technique to trap C-peptide oligomers (data not shown).

This crosslabeling technique is of value when trying to confirm an interaction between known interactants, and the MS-compatible workflow we developed in this study gains the method sensitivity. If dealing with unknown interaction partners the technique is probably sensitive enough to catch the interaction, but the identification could pose a problem as that requires sufficient amounts for analysis. The tag is sufficiently small to allow for intracellular capture of interacting partners.

PAPERS V-VI

C-peptide oligomerization

When trying to corroborate C-peptide nuclear localization by Western blot analysis, no C-peptide band at 3 kDa was observed. A long troubleshooting period followed, experimenting with transfer times, buffers and finally even equipment. Invitrogen launched a rapid dry-blotting system with which we observed C-peptide using a nitrocellulose membrane. As we used pure C-peptide solutions, it was obvious to us that the four bands observed must all be C-peptide. We showed that these oligomers form with native as well as Rh-labeled and biotinylated C-peptide, and that the oligomeric interaction is relatively salt-insensitive and heat-insensitive (**papers V-VI**). Furthermore, the aggregates formed by oligomers are shown to be of ß-sheet structure, and we suggested in **paper VI** that C-peptide is amyloid-like. Other studies have found C-peptide deposits in vivo (*76, 90*). As data on fibril formation is lacking, it does not qualify as an amyloid peptide (*91*).

In a previous study, C-peptide had been observed to disrupt insulin oligomers and to increase insulin bioavailability (*39*) but now we could show that insulin also disrupted C-peptide oligomers. This is interesting as Keltner et al. have shown that C-peptide is inactive by its ability to stimulate ATP release from erythrocytes prior to zinc binding (*92*). As insulin hexamers are stabilized by zinc and calcium (*40*), it is noteworthy that C-peptide upon insulin hexamer disaggregation releases zinc which can activate C-peptide and calcium which can act as a C-peptide oligomer disruptive agent.

C-peptide acts both to facilitate proper folding of proinsulin and to release insulin monomers, ensuring hormone bioavailability in accordance with the free hormone transport hypothesis (*93, 94*). The latter could also be true in reverse, as data evidence C-peptide oligomer disruption by insulin as well. These actions well represent a chaperone-function of C-peptide, as deduced by Wojcikowski et al. (*17*), Chen et al. (*95*), Shafqat et al. (*39*) and us (**paper V**).

OVERALL THOUGHTS

As the half-life of C-peptide far exceeds that of insulin (30 vs 5 min), it is reasonable to believe that C-peptide functions as a classical negative feedback loop molecule of insulin signaling pathways. This is supported by the fact that C-peptide inhibits insulin secretion (*16*), and also seems to regulate insulin bioavailability (*39*). However, there is also evidence of positive feedback loop mechanisms, as both C-peptide and insulin stimulate rDNA transcription via seemingly different mechanisms (*96*) (**paper II**), and insulinomimetic effects have been described in the literature (*25*). That C-peptide and insulin in addition must exert completely different effects is evident on the basis of different appearances, both in regard of structural invariability and variability as well as basic chemical properties.

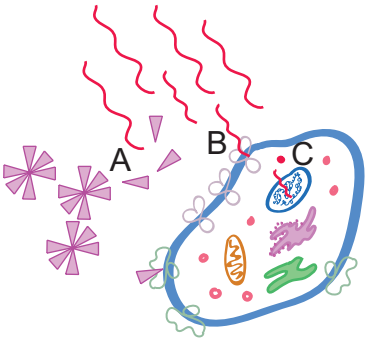

Figure 5. *Three-site activity model of C-peptide. (A) denotes C-peptide homo and C-peptide hetero interactions, (B) surface interactions with signaling cascade activation, and (C) intracellular and nuclear interactions.*

C-peptide exerts its activities in a compartmentalized fashion, extracellularly, at the cell surface and also intracellularly, Figure 5. To further specify these compartmentalized activities into tissue specific effects and to distinguish direct effects from secondary effects of C-peptide is a great challenge for future work. That a fine-tuned homeostasis of C-peptide is necessary for balanced effects is suggested by the long-term complications observed in type I diabetic patients. Evidence of long-term complications when reaching above normal physiological concentrations as in type II diabetes are also implicated. The sub- and supralevels of C-peptide observed in these diseases most likely affects the body in many ways, although a major distinc-

tion can be made. Sub-levels of C-peptide primarily seem to affect cellular function disturbing signaling pathways, whereas supra-levels are indicated to participate in aggregate formation. This remains to be further researched, as studies of C-peptide and type II diabetes primarily have involved measurements of insulin levels.

CONCLUDING REMARKS

Doing research on C-peptide has been a fascinating, educating, frustrating and creative journey. When I started I barely knew what C-peptide was, and today I find it difficult to leave this little peptide behind. Writing this thesis has given me the opportunity to observe new important stones still left unturned. I truly wish that C-peptide or an analogue will find a way into the clinic, and help patients suffering from type I diabetes complications.

If I were to commence on a graduate program on C-peptide function with the knowledge I have today, I would

- investigate C-peptide at its site of synthesis, i.e. how it interacts with different cell types in the Langerhans islets,
- continue on methodological work to allow for detailed analysis of C-peptide interactions in the nucleus,
- map C-peptide effects in one readily available model system to avoid inference of cell type and signaling pathway accessibility,
- collaborate with clinicians and get my hands on as much data as possible when clinical trials of C-peptide supplementation are available.

Now this is not the end. It is not even the beginning of the end. But it is, perhaps, the end of the beginning.

Sir Winston Churchill

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I would maintain that thanks are the highest form of thought; and that gratitude is happiness doubled by wonder.
G.K. Chesterton

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