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Development of long-acting recombinant glycoprotein hormones by increasing the carbohydrate content

Fuad Fares[1] and Naiel Azzam[1,2]

[1] Department of Human Biology, Faculty of Natural Sciences, University of Haifa, Haifa 3498838, Israel
[2] Migal-Galilee Research Institute, Kiryat Shmona 11016, Israel

Therapeutic recombinant glycoproteins are important for both the biotechnological industry and clinical purposes. Given the rapid clearance of these proteins from the circulation, they have to be injected frequently to obtain optimal therapy. Several strategies have been developed to overcome this limitation, aiming to increase the half-life of such proteins in the circulation. These strategies included chemical attachment of polyethylene glycol, nanocapsulation, fusion to immunoglobulins or to albumin as protein carriers, or enrichment of the carbohydrate content. Here, we describe a strategy for increasing the half-life of recombinant proteins using gene fusion to increase the carbohydrate content of the protein backbone.

Introduction

The development of therapeutic recombinant proteins is important for both the biotechnological industry and clinical applications. The US Food and Drug Administration (FDA) has approved more than 180 therapeutic recombinant proteins and peptides for the treatment of many diseases. Many recombinant proteins require post-translational modifications, such as glycosylation, for their function. Therefore, these proteins need to be expressed in eukaryotic cells, such as yeast, insect, and mammalian cells [1–4].

Most of these proteins are <50 kDa and, thus, are filtered quickly from the circulation, exhibiting a short half-life. There are several mechanisms involved in the clearance of recombinant proteins from the circulation, including proteolysis, renal filtration, and cleavage by hepatic enzymes [5,6]. Therefore, these proteins need to be injected frequently in clinical protocols to obtain optimal therapy. Thus, the development of long-acting recombinant proteins for the treatment of human diseases will diminish the cost of these drugs and perhaps reduce the number of injections required. To overcome the limitation resulting from a short half-life, several strategies have been used to increase the half-life of recombinant proteins in the circulation, such as chemical attachment of polyethylene glycol (PEGylation) [7–9], nanocapsulation [10,11], fusion to immunoglobulin-binding domains [12], conjugation to albumin as a carrier for peptide-based drugs [13,14], or by enrichment of the carbohydrate content of the protein backbone [15,16].

Here, we describe a strategy that could have wide application for enhancing the *in vivo* half-life of diverse proteins. We highlight the potential and challenges of this strategy for developing long-acting recombinant proteins, and emphasize the advantages of this technology among the pre-existing methods available.

The role of oligosaccharides in the bioactivity, half-life, and immunogenicity of recombinant glycoprotein hormones

Glycosylation is one of the most frequent post-translational modifications of extracellular and secreted proteins. The addition and processing of oligosaccharide chains on a protein can affect the efficient intracellular routing and secretion of the protein.

Human chorionic gonadotropin (hCG)β subunit contains an *O*-linked-rich hydrophilic C-terminal extension peptide (CTP) of 28 amino acids, which is not found in the other subunits of glycoprotein hormones. Crystal structure studies predicted that the CTP does not fold with the hCGβ subunit (Fig. 1). It appears that the amino acid sequence of CTP, marked in red in Fig. 1a, does not fold

Corresponding author: Fares, F. (ffares@sci.haifa.ac.il)

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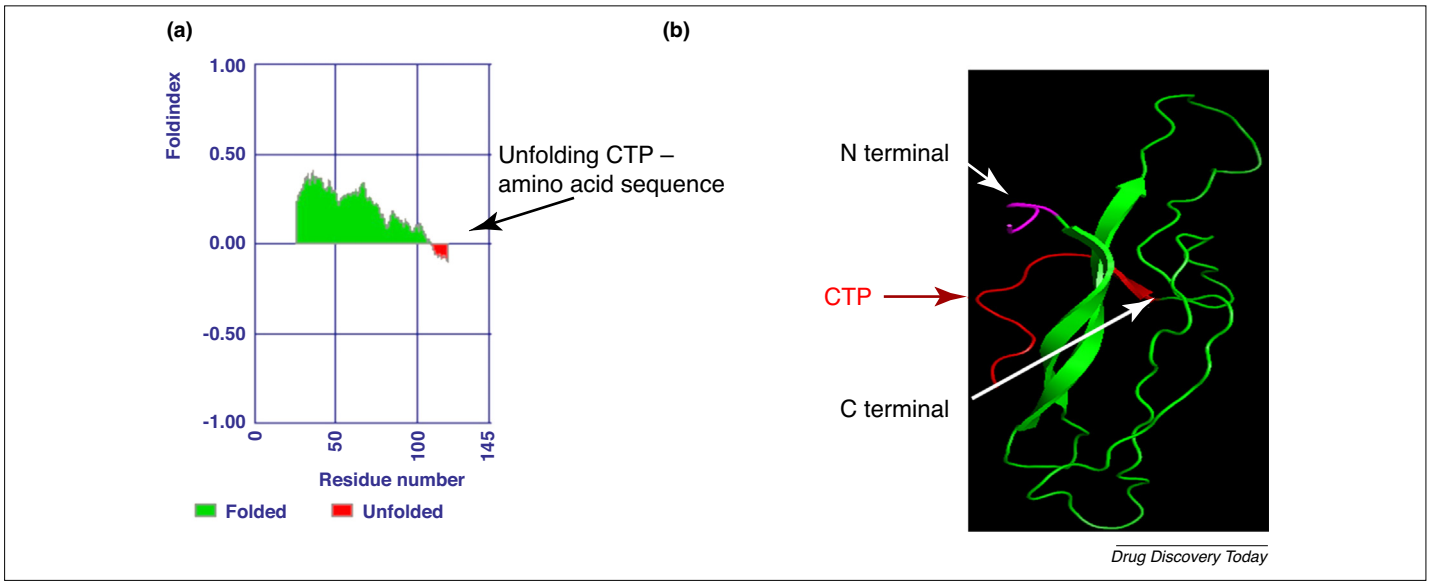

FIGURE 1

Prediction of folded and unfolded regions of the human chorionic gonadotropin (hCG)β chain. The amino acid region of C-terminal peptide (CTP) marked in red (a) does not fold with hCGβ amino acids 1–115 (in green). In addition, the crystal structure analysis (b) indicated that the CTP sequence at the C-terminal end hovers outside of the folded hCGβ subunit.

with amino acids 1–115 of the hCGβ (in green in Fig. 1a). In addition, crystal structure analyses indicated that the CTP sequence hovers out of the folded hCGβ subunit (Fig. 1b). To examine the role of *O*-linked oligosaccharides and the unique C-terminal extension of the hCGβ subunit in receptor binding and bioactivity, two strategies were described. The first strategy was the expression of hCG in mutant Chinese hamster ovary (CHO) ldlD cells, which are UDP-galactose (Gal)/UDP-N-acetylgalactosamine (GalNAc) 4-epimerase deficient. This deficiency prevents synthesis of *N*-acetylgalactosamine (GalNAc), which is needed for the addition of *O*-linked oligosaccharide chains to the amino acids of serine/threonine [17]. The second strategy was the design of a mutant *hCGb* gene that contains a premature termination signal at codon 115 upstream of the CTP sequence (Fig. 2), forming hCGβ lacking the CTP sequence [18]. Results of these studies indicated that the CTP of the hCGβ subunit and the associated *O*-linked oligosaccharides are not important for receptor binding or *in vitro* signal transduction, but are crucial for *in vivo* biological responses.

Both *N*-linked and *O*-linked oligosaccharides are frequently terminated with sialic acids. Sialic acids have an important role in molecular and cellular interactions. Interestingly, the sialic acids on oligosaccharide surfaces create a high negative charge on secreted proteins, suggesting that they have a role in the half-life extension of glycoproteins in circulation. This might be related to decreases in kidney glomerular filtration [19,20].

By contrast, upon loss of sialyation, proteins can be recognized by macrophages and hepatocytes, via Gal-recognizing receptors, and are taken up and enzymatically degraded. Therefore, attachment of sialic acid can protect proteins from proteases and glucosidases, resulting in extension of their lifetime and function *in vivo* [21,22]. Thus, the presence of sialated *O*-linked oligosaccharides might prolong the circulating half-life of the hCG by preventing the effects of liver enzymes and resulting in a decrease in renal clearance.

In general, treatment with modified recombinant proteins can result in the development of immune responses. This issue remains the most serious complication in replacement therapy. Theoretically, ligation of CTP to different proteins forms new compounds that could be foreign to the human immune system. Thus, the possible antigenicity of these chimeras has been of primary concern. Previously, it was reported that the CTP region of hCG is not immunogenic [23]. Clinical studies using FSH-CTP

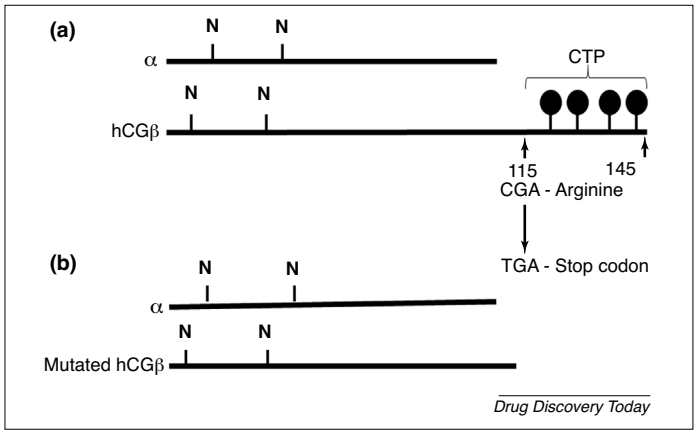

FIGURE 2

Designing a human chorionic gonadotropin (hCG) variant lacking the C-terminal peptide (CTP) by site-directed mutagenesis. The codon at amino acid 115 of CGA (arginine) was mutated to a stop codon (TGA) to produce a truncated hCG dimer lacking the C-terminal amino acids 115–145 of hCGβ subunit. The wild-type and mutant hCG were expressed in Chinese hamster ovary (CHO) cells and tested *in vitro* and *in vivo*, with no effect on receptor binding and signal transduction indicated, but having a crucial role in *in vivo* bioresponses.

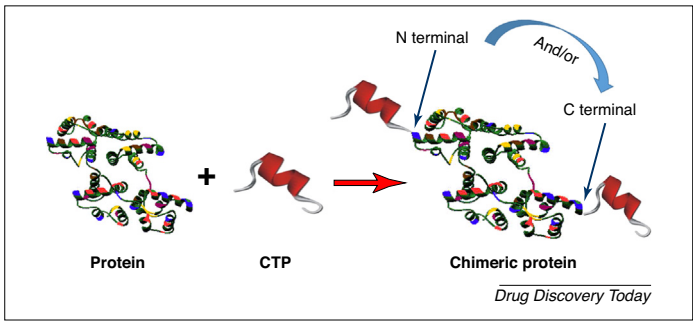

FIGURE 3

Ligation of the C-terminal peptide (CTP) sequence to the C and/or N terminal of a protein can increase its half-life *in vivo*. The coding sequence of the human chorionic gonadotropin (hCG)β C-terminal peptide containing the recognition sites of *O*-linked oligosaccharides can be ligated to the 5′ end and/or to the 3′ end of a gene to produce long-acting chimeric proteins.

and GH-CTP indicated that no antibodies against these hormones were detected [24–26]. Possibly, the *O*-linked oligosaccharides attached to the CTP act as mask covering the antigenic sites of the protein. This could shed light on the mechanism of how the addition of *O*-linked oligosaccharides to the backbone of the protein prevents immunogenic effects [27]. Moreover, because the native hCG that contains the CTP is normally secreted in both men and women, the immune system might not recognize chimeras containing CTP as a foreign protein [23]. This might explain the findings that ligation of CTP bearing four *O*-linked oligosaccharides ending with sialic acid to different proteins, such as follicle-stimulating hormone (FSH) and growth hormone (GH), is not immunogenic and results in a significant increase in the half-life of proteins in circulation without exhibiting any immunogenicity. Thus, the strategy of adding a CTP sequence to the coding genes of recombinant proteins (Fig. 3) appears to be relevant to increasing their half-life and bioactivity *in vivo* .Using this strategy, we describe here the development of long-acting FSH, GH, and erythropoietin (EPO) recombinants.

Developing long-acting recombinant proteins

Follitropin

Follitropin (FSH) is a gonadotropin secreted from the anterior pituitary and is required for normal reproductive function in both males and females. FSH together with lutropin (luteinizing hormone; LH), hCG, and thyrotropin (thyroid-stimulating hormone; TSH) comprise the glycoprotein hormone family. These hormones are heterodimers comprising two subunits, α and β, where the α subunit is common and the β subunit is specific. FSH is clinically used to stimulate ovarian follicular development for *in vitro* fertilization (IVF) to obtain a higher number of oocytes [28,29]. In males, FSH is crucial for regulating and maintaining spermatogenesis, in that sperm ultrastructure and motility improvements were observed following FSH administration [30]. Follitropin was previously purified from urine of older women for clinical use [31]. However, the identification of *FSHb* and *FSHa* genes enabled the development of DNA technology to produce recombinant FSH in mammalian cells for human use [32].

FSH has to be injected daily because of its relatively short half-life. Therefore, there is a need to develop long-acting recombinant FSH to reduce the number of administrations. To overcome this issue, the sequence of CTP of hCGβ was ligated to the coding sequence of the FSHβ subunit (Fig. 3). This construct was co-transfected into CHO cells together with an α subunit, using mammalian expression vectors (Fig. 4a), to produce a dimeric recombinant hormone (Fig. 4b). The results indicated that ligation of one CTP to the C terminal of FSHβ had no effect on the assembly of the subunits, folding of the protein, receptor binding affinity, or *in vitro* bioactivity. However, FSH-CTP showed a longer half-life and higher bioactivity *in vivo* [15,33,34]. The first human exposure to FSH-CTP by treatment of hypogonadotropic males indicated that this construct is safe for use and not immunogenic [35]. These observations were important for further use of FSH-CTP for human IVF protocols. According to the IVF protocol, women are treated once with FSH-CTP, followed after Day 7 by daily doses of wild-type (WT) recombinant FSH (rhFSH-WT), until Day 14 of the menstrual cycle to trigger final oocyte maturation. This protocol indicates that a single-dose administration of FSH-CTP is a

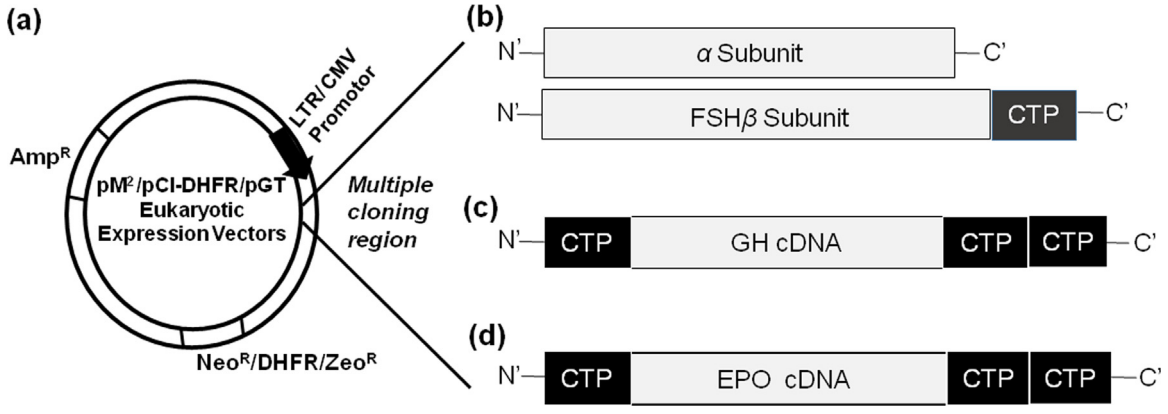

FIGURE 4

Schematic presentation of the eukaryotic expression vectors (pM^2, pCI-DHFR, pGT) (a) respectively used for cloning of follicle-stimulating hormone (FSH) (b), growth hormone (GH) (c), and erythropoietin (EPO) (d) variants containing the sequence of human chorionic gonadotropin (hCG)β C-terminal peptide (CTP). CTP sequence was ligated to the carboxyl-terminal of FSHβ subunit and to N- terminal and C- terminals of GH and EPO. The chimeric genes were cloned into the multiple cloning regions of the expression vectors under the LTR promoter in pM2 and the CMV promoter in pCI-DHFR and pGT vectors. Cloned vectors were transfected into Chinese hamster ovary cells (CHO) for recombinant protein production [33,53,59].

potent inducer of follicular growth compared with daily treatment with rhFSH. Furthermore, the terminal half-life of FSH-CTP in circulation was 65 h, two to three times longer than that of rhFSH-WT [35–40]. These observations revealed that a single subcutaneous injection of FSH-CTP can replace seven daily injections of FSH-WT during the first week of controlled ovarian stimulation, making assisted reproduction more patient friendly. FSH-CTP was approved by the European Commission (EC) for controlling ovarian stimulation and for use in IVF protocols, and is marketed by Merck as ELONVA.

Other researchers described an alternative method for increasing the half-life of rhFSH by introducing new *N*-linked glycosylation sites using site-directed mutagenesis [41,42]. The results indicated that the addition of *N*-linked carbohydrate chains did not affect the folding of the hormone or the binding affinity to its receptor. By contrast, it increased the half-life and bioactivity *in vivo* in animal models. This strategy might change the structure of FSH and might trigger an immune response in the body. These analogs were not tested in clinical studies and their immunogenicity profile is not known.

Growth hormone

GH is a single-chain polypeptide that is secreted from somatotropic cells within the anterior pituitary gland. GH stimulates growth and cell generation and it is important for human development. It is used clinically to treat growth disorders in children and GH deficiency in adults [43–46]. The first source of GH in clinics was extractions from the pituitary gland of cadavers. In 1985, the FDA approved recombinant GH (rhGH) produced in *Escherichia coli* for the treatment of GH deficiency (GHD) in childhood. Following this approval, rhGH replaced pituitary-derived human GH for therapeutic use. Long-term trials indicated the efficacy and safety of rhGH therapy. Over the past two decades, such therapies have been expanded. Today, rhGH is used to treat adult GHD (AGHD) [43–46], as well as Turner's [47] and Prader–Willi [48] syndromes. Studies showed an increase in velocity of height gain after 6–12 months of rhGH replacement. The major obstacle in the clinical use of GH is the notable short half-life in circulation and its rapid clearance ($T_{1/2}$ ~12 min) [49]. Therefore, GH was injected frequently to reach the optimal bioactivity. Thus, efforts have focused on developing a long-acting rhGH. The rational was to stabilize GH in the circulation, enabling administration of a once weekly instead of daily injections. In recent years, several strategies for developing long-acting GH have been described. It was found that GH could be protected from degradation through covalent binding to PEG molecules [50,51]. Free PEG molecule is soluble in water and organic solvents, lacks immunogenicity, increases protein conformational stability, harbors enhanced pharmacokinetic properties, and is easily cleared from the body via the kidney and liver [52]. Therefore, PEGylation of rhGH reduced its renal clearance and increased its half-life in circulation. Furthermore, weekly administered PEG-rhGH is safe and effective in the treatment of GHD. Although PEGylation remains an excellent choice for protein stabilization and controlled dosage of protein drugs, it still has several limitations. For instance, protein PEGylation should avoid active sites and binding interfaces in addition to its high manufacturing costs.

Another strategy to developing long-acting rhGH has also been described. Human serum albumin (HSA) was genetically fused to recombinant hGH (TV-1106). Single administration of TV-1106 was safe in healthy volunteers, while clinical studies support once-weekly administration in patients with GHD.

A long-acting GH analog was also developed by fusing the CTP to the N- and C-terminal sequences of GH (Fig. 4c). The N- and C-terminal ends of GH are not involved in receptor binding [15] and, therefore, it was hypothesized that ligation of CTP sequence to these ends would not affect receptor-binding affinity and bioactivity and might increase the half-life in circulation. Different analogs of hGH were prepared by fusing one or more CTPs to the N or C terminal of GH [53]. The results showed that ligation of one CTP cassette to the N terminal and two cassettes to the C terminal of hGH (MOD 4023) resulted in a significant increase in the half-life in circulation. Moreover, treatment of hypophysectomized male mice twice, once every 5 days with MOD-4023, resulted in a dramatic increase in weight gain and was found to be safe [49,53]. In addition, treatment with MOD-4023 every 5 days significantly increased weight gain in rats and monkeys. These results provide a basis for the clinical development of MOD-4023 as a novel treatment of GHD in children and adults. Clinical studies Phase II in adult patients with GHD found this substance to be effective and safe. Moreover, weekly MOD-023 injections also improved compliance and overall outcome [54,55]. These studies have led to a Phase III trial that is currently ongoing.

Therefore, it appears that treatment once weekly with long-acting rhGH represents an advance over daily injections of rhGH-WT. Moreover, it also appears that rhGH is safe for use, not immunogenic, and increases the quality of life for patients.

Erythropoietin

EPO is a glycoprotein hormone secreted by kidneys in response to cellular hypoxia. It stimulates red blood cell production (erythropoiesis) in the bone marrow [56]. Although EPO is mainly produced and secreted from fetal liver and adult kidney, it was also reported that it is locally produced and released by cells of various tissues, including heart, spleen, lung, testis, ovaries, retina, and the nervous system. In addition, it was reported that EPO has a role in angiogenesis in endothelial cells in heart, brain, and leg ischemia, as well as in retinopathy protection and tumor promotion.

EPO is a single-chain glycoprotein with 165 amino acids and is post-translationally modified to include three *N*-linked and one *O*-linked oligosaccharide chains. It binds to specific receptors on the surface of red blood cell precursors in the bone marrow, leading to their survival, proliferation, and differentiation, and ultimately to an increase in hematocrit.

Epoetin alfa is a recombinant human EPO (rhEPO) that was produced by recombinant DNA technology in CHO cells. rhEPO has become the standard of care in treating anemia associated with chronic renal failure (CRF). In addition, benefits of using high-dose rhEPO for its nonhematopoietic function as a cytokine that enhances tissue repair after injury have also been reported. These studies demonstrate clear evidence that administration of rhEPO near the time of injury in experimental acute kidney injury (AKI) and acute myocardial infarction (AMI) significantly improved recovery. This effect of EPO included inhibition of apoptosis, stimulation of mitogenesis and cell differentiation, mobilization of endothelial cells, angiogenesis, and suppression of proinflammatory cytokine mediators [56].

The recommended therapy with rhEPO is two to three times per week by subcutaneous or intravenous injection. Therefore, there is a need to develop a long-acting analog that can be used just once a week. For this purpose, a hyperglycosylated rhEPO was designed by the addition of two *N*-linked carbohydrate chains to the backbone of EPO. To introduce a new *N*-linked oligosaccharide chain, the DNA sequence of the gene encoding EPO was modified by site-directed mutagenesis to add the consensus sequence for carbohydrate addition (Asn–X–Ser/Thr). The developed analog (ARANESP) contains two more *N*-linked carbohydrate chains [57,58]. These studies showed that ARANESP has an approximate threefold longer serum half-life, greater *in vivo* potency, and can be administered less frequently to obtain the same biological response, compared with repeated administration of rhEPO-WT. However, the receptor-binding affinity of ARANESP is significantly reduced.

Previously, based on crystal structure studies of EPO and its receptor, the N- and C-terminal ends of EPO were found not to be involved in receptor binding [15]. Thus, CTP was ligated to the C terminal of EPO, resulting in an analog that bound to the receptor with high affinity, leading to a significant increased bioactivity *in vivo* [59]. Then, one and two CTP sequences were ligated to the N and C terminals of human EPO coding sequence, respectively (Fig. 4d). This analog was expressed in CHO cells, secreted to media, and was found to bind to the EPO receptor with high affinity and to be biologically active *in vitro*. Interestingly, this analog was also more stable and had a longer half-life *in vivo* compared with rhEPO-WT or ARNASEP. Moreover, using animal models, it was revealed that treatment of EPO-CTP once weekly dramatically increased the bioactivity compared with three injections of rhEPO a week [15,59,60]. This EPO analog also appears safe but needs to be tested in clinical trials.

Concluding remarks

Based on the above reviewed technologies used for the development of stable, safe, and long acting recombinant glycoproteins, the advantages of fusing CTP to the protein edges include: (i) the modification is carried out at the level of the coding gene; (ii) ligation of CTP to the coding sequence of the protein increases its secretion from cells, enabling high levels of production; (iii) CTP, which was deprived from hCG, is of human origin and therefore, not immunogenic; and (iv) CTP does not participate in protein folding when ligated to the N or C terminal of the designed proteins and, therefore, has no effect on the conformation of the protein or on receptor-binding affinity or bioactivity *in vitro*, but increases the longevity *in vivo*.

Industrial biotechnology using molecular methods has become the main strategy for the production of recombinant proteins. The progress in the field of recombinant technology and biotechnology now enables the production of a large number of peptides and proteins in commercial quantities. The main problem associated with using these proteins in the clinic is their short half-life in circulation because of fast renal clearance and enzymatic degradation in the liver. Therefore, these proteins are injected frequently to sustain bioactivity. To overcome this issue, different methods have been used to stabilize the protein molecules, reduce their clearance from the circulation, and increase their half-life. The stabilizing methods included nanocapsulation, chemical modification with polymers, and introducing oligosaccharide chains to the backbone of the proteins. The strategy of ligating the CTP peptide, which contains four sites of *O*-linked oligosaccharides, to the coding sequence of proteins appears to be a particularly useful approach. Ligation of one or more CTPs to the coding sequence of proteins, while avoiding the domains that are important for receptor binding or bioactivity, has no effect on receptor-binding affinity, is not immunogenic and significantly increases the longevity *in vivo*. Proteins that are developed by fusion of one CTP (FSH) or three CTPs (GH and EPO) appear to be safe and can be injected only once a week. Thus, this strategy could be used as a platform for the further development of long-acting recombinant proteins.

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