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Design, recombinant expression and in vitro maturation of human insulin-like peptide 6 and a biotin-labeled analogue

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ABSTRACT

Insulin-like peptide 6 (INSL6) is a newly identified peptide hormone belonging to the insulin/relaxin superfamily. It is predominantly expressed in the testes and responsible for spermatogenesis. It also shows potential for the treatment of benign prostate hyperplasia. For further basic and preclinical studies, significant quantities of INSL6 peptide are needed. In the present work, we designed and recombinantly expressed a single-chain human INSL6 precursor in *Escherichia coli*. After purification, in vitro refolding, and cyanogen bromide cleavage, the single-chain precursor was converted to mature two-chain INSL6 peptide. So far, the receptor of INSL6 is remained unknown. To identify its receptor, site-specifically biotin-labeled INSL6 analogues are useful. Thus, we designed and recombinantly prepared a two-chain INSL6 analogue carrying an Avi-tag at the N-terminal of B-chain. Subsequently, biotin moiety was covalently attached to the Avi-tag of the analogue catalyzed by biotin ligase. Circular dichroism spectroscopy suggested that the recombinant INSL6s adopted insulin-like fold. Our present work provided an efficient approach for the preparation of the recombinant INSL6 peptide and its analogues for further studies of this important peptide.

1. Introduction

Insulin superfamily is a group of peptide hormones that share an identical cysteine arrangement pattern but which have diversified biological functions [1]. The human genome totally encodes 10 insulin superfamily members, including insulin, insulin-like growth factors 1 and 2 (IGF-1 and IGF-2), insulin-like peptide 3–6 (INSL3, INSL4, INSL5, and INSL6), and relaxin 1–3. Among these, insulin-like peptide 6 (INSL6) was first identify from the Expressed Sequence Tag database based on the insulin family B-chain cysteine motif in 1999 [2–4], it is also named relaxin/insulin-like factor 1 (RIF1) [2]. Human insl6 gene is mapped to chromosome 9p24 near the insulin-like peptide 4 (INSL4) and the autosomal testis-determining factor locus [3]. INSL6 is predominantly expressed in the testes [2–5], and the expressed INSL6 peptide can be detected in vivo and in cultured cells [6]. A recent insl6-knockout experiment demonstrated that INSL6 is required for the progression of spermatogenesis in mice [7]. The majority of the insl6-knockout male mice exhibit impaired fertility, whereas the female knockout mice are fertile [7]. INSL6 also shows potential for the treatment of benign prostate hyperplasia, a disease affecting high percentage of elder men (US patent, US7314911B2). So far, the receptor of INSL6 is remained unknown.

For further basic and preclinical studies, significant quantities of mature INSL6 peptide are needed. Meanwhile, biotin-labeled INSL6 analogues will be helpful for INSL6 receptor-screening from cDNA library or receptor purification from tissue resource. In the present work, we established a highly efficient approach for the preparation of both mature and biotin-labeled INSL6 peptides.

2. Materials and methods

2.1. Materials

The oligonucleotide primers were chemically synthesized at Biosune (Shanghai, China). The Agilent reverse-phase columns (analytical column: Zorbax 300SB-C18, 4.6 mm × 250 mm; semi-preparative column: Zorbax 300SB-C18, 9.4 mm × 250 mm) were used in the experiments. The peptide was eluted from the reverse-phase column by an acetonitrile gradient composed of solvent A and solvent B. Solvent A was 0.1% aqueous TFA, and solvent B was acetonitrile containing 0.1% TFA. The elution gradient was listed as follows: 0 min, 10% solvent B; 3 min, 10% solvent B; 53 min, 60% solvent B; 55 min, 100% solvent B; 56 min, 100% solvent B, 60 min, 10% solvent B. The flow rate for analytical column was 0.5 ml/min, and that for semi-preparative column was 1.0 ml/min. The eluted peptide was detected by UV absorbance at both 280 nm and 214 nm.

Abbreviations: CD, circular dichroism; CNBr, cyanogen bromide; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; GSSG, oxidized glutathione; HPLC, high performance liquid chromatography; IPTG, isopropyl β-D-thiogalactoside; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; TCEP, tris(2-carboxyethyl)phosphine; TFA, trifluoroacetic acid; UV, ultra-violet.

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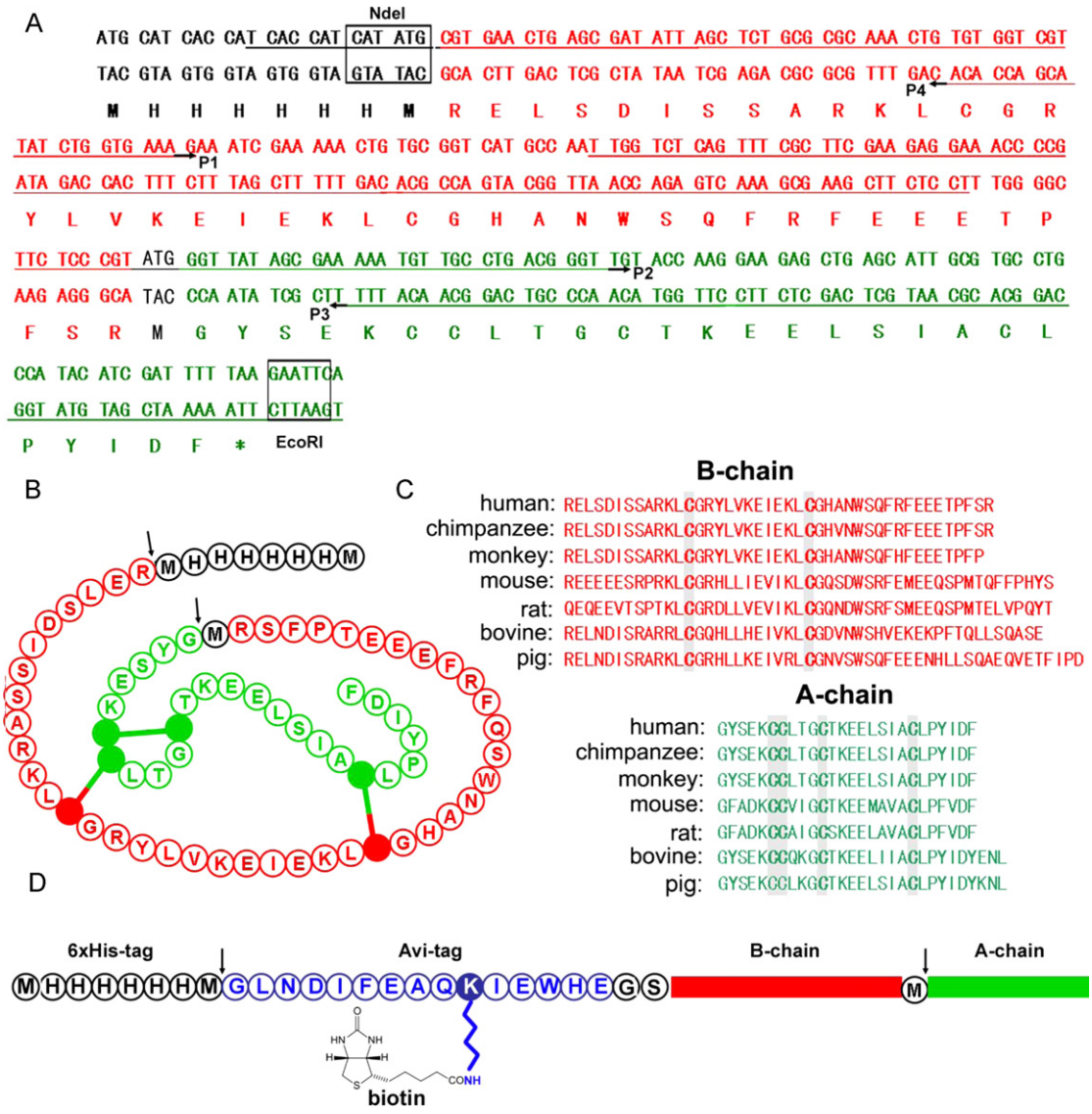

Fig. 1. (A) The amino acid sequence and nucleotide sequence of 6×His-INSL6. The B-chain and A-chain are shown in red and in green, respectively. The N-terminal 6×His-tag and the linker Met between B- and A-chains are shown in black. Four oligonucleotide primers (P1, P2, P3, and P4) used to construct the gene of 6×His-INSL6 are underlined and labeled. The restriction enzyme cleavage sites (NdeI and EcoRI) are also labeled. (B) A cartoon show of the amino acid sequence of 6×His-INSL6. The cysteines are shown in filled circles and the disulfide bonds are shown as sticks. The CNBr cleavage sites are indicated by arrows. (C) The amino acid sequence of INSL6s from different species. (D) A cartoon show of the sequence characteristics of 6×His-Avi-INSL6. The biotin moiety covalently attached to the Avi-tag (blue) after enzyme-catalyzed biotin-labeling is shown. The CNBr cleavage sites are indicated by arrows. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2.2. Gene construction, recombinant expression, and purification of INSL6 precursors

The encoding DNA fragment of 6×His-INSL6 was constructed from 4 chemically synthesized oligonucleotide primers, and that of 6×His-Avi-INSL6 was constructed from 6 oligonucleotide primers. The encoding DNA fragment was cleaved by restriction enzymes NdeI and EcoRI, and subsequently ligated into a pET expression vector. The encoding DNA sequence was confirmed by DNA sequencing.

The expression construct was transformed into *Escherichia coli* strain BL21(DE3)star. The transformed *E. coli* cells were cultured in liquid LB medium (with 100 μg/ml ampicilin) to $OD_{600\,nm}$ = 1.0 at 37 °C with vigorous shaking (250 rpm). Then IPTG stock solution was added to the final concentration of 1.0 mM, and the cells were continuously cultured at 37 °C for 5–6 h with gentle shaking (100 rpm).

The *E. coli* cells were harvested by centrifugation (5000 × *g*, 10 min), resuspended in lysis buffer (20 mM phosphate buffer, pH 7.4, 0.5 M NaCl), and lysed by sonication. After centrifugation (18,000 × *g*, 30 min), the pellet was solubilized by solubilizing buffer (lysis buffer plus 8 M urea). After centrifugation (18,000 × *g*, 30 min), the supernatant was loaded onto a Ni^{2+} column that was pre-equilibrated with the solubilizing buffer. The precursor was then eluted from the column by step-wise increase of imidazole concentration in the solubilizing buffer. The eluted INSL6 precursor fraction was manually collected, and used for in vitro refolding.

2.3. In vitro refolding of the INSL6 precursors

DTT stock solution was added to the above eluted INSL6 precursor fraction (peptide concentration ~3.5 mg/ml) to the final concentration of 20 mM in order to reduce the disulfide bonds. The reduction reaction was carried out at room temperature for 1 h. Thereafter, the reduced INSL6 precursor was 20-fold diluted into the pre-warmed refolding solution (0.5 M L-arginine-HCl, 1.0 mM EDTA, 2.0 mM GSSG, pH 8.5). The refolding was carried out at 16 °C for 2–3 h. Subsequently, the refolding mixture was acidified to pH 2.0 and subjected to C18 reverse-phase HPLC. The refolded peptide was eluted from the column by an acetonitrile gradient as listed in Materials. The eluted precursor was manually collected, lyophilized, and analyzed by mass spectrometry.

2.4. Chemical cleavage of the INSL6 precursors

The refolded INSL6 precursor was dissolved in the fresh cleavage solution (0.1 M HCl, 5 M guanidine chloride, 200 mg/ml CNBr) at the final concentration of ~2 mg/ml. The chemical cleavage was carried out at 4 °C for 4 h. Subsequently, the cleavage