Carassius auratus-Originated Recombinant Histone H1 C-Terminal Peptide as Gene Delivery Material

Hee Jung Jung,†,‡ Dong Soo Hwang,†,‡ Quan De Wei,†,§ and Hyung Joon Cha*†

Department of Chemical Engineering, Pohang University of Science and Technology, Pohang 790-784, Korea, and Center for Disease Control and Prevention of Zhuhai, Zhuhai 519000, People’s Republic of China

The effective delivery of exogenous genes into eukaryotic cells is important for fundamental and biotechnological research. Protein-based gene delivery including histone proteins has recently emerged as a powerful technique for non-viral DNA transfer. Histones are DNA-binding proteins that function in DNA packaging and protection. In particular, histone H1 is largely responsible for the stabilization of higher-order chromatin structures. Several studies have examined the use of full-length histone H1-mediated gene transfer, and a few studies have investigated the use of C-terminal histone H1 fragments as gene-transfer materials. Previously, we cloned a novel histone H1 cDNA from the goldfish Carassius auratus and found that a recombinant histone H1 C-terminal short peptide (H1C) of 61 amino acids has comparable DNA binding and protection functions as full-length histone H1. In the present work, we successfully expressed and purified soluble recombinant H1C in an Escherichia coli expression system using a hexahistidine tag fusion strategy and providing tRNAs for rare codons. We confirmed its DNA-binding ability and found that this H1C peptide had similar or higher transfection efficiency in mammalian cells (human 293T and mouse NIH/3T3) than the widely used agent lipofectamine. Therefore, we suggest that this novel goldfish-derived recombinant histone H1 C-terminal short peptide could be used as a peptide-based gene-transfer mediator.

Introduction

The effective delivery of exogenous nucleotides into cells is a basic and important step in gene therapy and also has implications in fundamental and biotechnological research. In the future, gene transfer into eukaryotic cells may be used to treat many diseases that are currently considered incurable. Although there have been marked improvements in gene-delivery technologies based on cationic lipids and viral vectors in recent years, these strategies are still limited in terms of safety, immunogenicity, size of the target gene, specificity, production, toxicity, and cost (1). Recently, increased attention has been paid to non-viral gene-delivery systems, which have relatively low immunogenicity, as well as increased safety and simplicity compared with viral systems (2, 3). Such non-viral systems include the protein-based gene-transfer method, which has benefits including ease of use and production, homogeneity, the ability to target nucleic acids to specific cell types, no limit on the size or type of nucleic acid that can be delivered, the possibility of modular attachment of targeting ligands, and the potential for cost-effective, large-scale manufacturing. Several studies have investigated histone-mediated gene transfer as a potential protein-based method because histone proteins can condense and compact DNA through interactions with the negatively charged sugar–phosphate backbone (4–10).

Histones are octamers of small, basic proteins that are water-soluble and abundant in lysine and arginine residues. Histones are major components of chromatin and act as spools for DNA winding, thereby facilitating the compaction necessary to fit large eukaryotic genomes inside cell nuclei (11). In eukaryotes, transcriptional repression largely correlates with chromatin condensation. There are five types of histones: H1, H2A, H2B, H3, and H4. Unlike other histones, histone H1 is not involved in formation of the nucleosome histone octamer but is instead believed to be mainly responsible for condensation of the chromatin into the thick chromatin fiber (12). Histone H1 is associated with linker DNA and may be positioned at the point where DNA enters and leaves the nucleosome. Histone H1 molecules consist of three domains: a short, basic N-terminal domain; a highly conserved central globular domain; and a basic C-terminal domain (13). Recent experiments have demonstrated that the basic C-terminal domain is largely responsible for the ability of histone H1 to bind chromatin and mediate condensa-

Figure 1. Western blot analysis for expression of recombinant H1C peptide from E. coli Rosetta (DE3). Lanes: M, prestained protein molecular weight marker; C, whole-cell sample containing the parent vector pET22b+ (negative control); W, whole-cell sample; S, soluble supernatant fraction; IS, insoluble cell debris fraction. Recombinant proteins were cultured in LB medium at 37 °C and 250 rpm with ampicillin and chloramphenicol and induced by 1 mM IPTG for 6 h. 15% (w/v) gels were used for analysis.
tion (14–16). Several studies have examined the use of full-length histone-mediated gene transfer, whereas only a few studies have shown that the use of C-terminal histone fragments can also affect gene-transfer efficiency (7, 10).

Previously, we have investigated expression of a functional novel histone H1 protein from the goldfish Carassius auratus (C. auratus) in Escherichia coli (E. coli) using the maltose-binding-protein fusion strategy (17). Interestingly, we found that the 61 amino acids at the C-terminal (H1C) of histone H1 (183 nucleotides) have similar DNA-binding and protection abilities, that is, a prerequisite for gene transfer, compared with full-length histone H1 protein. In the present work, we expressed and purified C. auratus-derived H1C peptide in E. coli using a hexahistidine (His6) fusion strategy and supplementing with tRNAs for rare codons, and investigated the potential of purified recombinant H1C peptide as a DNA-delivery material.

Materials and Methods

Construction of Recombinant Plasmid. The cDNA coding for the C-terminal sequence (61 amino acids) of histone H1 from C. auratus was amplified from the recombinant pMHC plasmid (17) as a template by polymerase chain reaction (PCR) using the primers (forward, 5′-GCCATATGGGCAAGAGCG-3′; reverse, 5′-GGCTCGAGGCTTTGCTGTCT-3′). The resulting 183 bp PCR fragment was inserted into the NdeI and XhoI sites of the pET-22b(+) vector (Novagen) that contains an IPTG-inducible T7lac promoter and His6 tag at the C-terminus to generate pET-H1C (5.5 kb). The nucleotide sequence of the inserted gene was verified by direct sequencing.

E. coli Strains and Culture Condition. E. coli TOP10 [F− mcrA Δ(mrr-hsdRMS-mcrBC) Δ(lacY1Δ16lacZΔM15 lacX74 deoR recA1 araD139 (ara-levU7697 galU galK rpsL (Strr) endA1 nupG) [Invitrogen] was used for construction of recombinant plasmid. E. coli Rosetta (DE3) [F− ompT hsdSB(rB− mB−) gal dcm (DE3) pRARE2 (ComR)] (Novagen) was used for expression of recombinant H1C peptide. For construction of recombinant plasmid, E. coli cells were grown in Luria–Bertani (LB) medium. For production of recombinant H1C peptide, seed cells were grown in LB medium with ampicillin (50 mg/mL) and chloramphenicol (50 mg/mL) as selection pressures for plasmid-harboring strains in a 250 mL flask at 37 °C with 250 rpm. Seed culture was transferred (1%, v/v) to a final working volume of 4 L in a 9 L bioreactor (KoBiotech). Cell growth was monitored by measuring the optical density at 600 nm (OD600) using a UV–vis spectrophotometer (Shimadzu). When cultures reached an OD600 = 0.5–0.6, 1 mM (final concentration), IPTG (Sigma) was added to the culture broth for induction of recombinant H1C peptide. After 6 h induction at 37 °C, the postinduction cultures were harvested for subsequent purification.

Purification of Recombinant H1C. Recombinant H1C peptide fused with His6-affinity ligand was purified by immobilized metal affinity chromatography (IMAC). Cells were harvested by centrifugation at 4000 rpm for 20 min. Harvested cell pellets were resuspended in 5 mL of IMAC buffer, pH 8.0 (8 M urea, 10 mM Tris-HCl, 100 mM sodium phosphate), per gram (wet weight). Samples were lysed by shaking incubator for 2 h at 37 °C, lysates were centrifuged at 8000 rpm for 20 min at 4 °C, and the supernatant was collected for purification. IMAC purification was performed using the Acta Prime Purification System (Amersham Biosciences) at room temperature at a rate of 1 mL/min. Nickel–nitritotriacetate agarose (Qiagen; 7 mL) charged with 0.1 M NiSO4 was used as an affinity purification resin. The column was equilibrated with five rinse volumes of IMAC buffer with pH 8.0. After loading of denatured cell lysates, the column was consecutively washed with IMAC buffers at pH 6.3 and pH 5.9. Target recombinant H1C was eluted in IMAC buffer pH 4.5. The eluate was collected in 3 mL fractions, the UV absorbance at 280 nm of 70 fractions was analyzed, and the fractions were then separated by 15% (w/v) Tricine sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Finally, eluted recombinant H1C protein samples were dialyzed in buffer (20 mM Tris-HCl, 0.5 M NaCl, 0.1% Tween-20, pH 8.0). After dialysis of the H1C peptide, total protein amounts were quantified using a total protein assay kit (Bio-Rad).

SDS-PAGE and Western Blot Analysis. Culture samples were centrifuged at a maximal speed (13 000 rpm) for 10 min at room temperature. The cell pellets in protein sample buffer (0.5 M Tris-HCl (pH 6.8), 10% glycerol, 5% SDS, 5% /β-mercaptoethanol, and 0.25% bromophenol blue) and heated to 100 °C for 5 min. After centrifugation for 1 min, the samples were loaded onto a 15% (w/v) Tricine SDS-PAGE. The gels were colored by Coomassie blue staining (Bio-Rad) and were Western blotted. For Western blot analysis, proteins were transferred onto a nitrocellulose membrane (Sheleicher & Schuell BioScience) using a semi-dry blotting system (Amersham Pharmacia) and Bjerrum and Schafer-Nielsen transfer buffer (48 mM Tris, 39 mM glycine, 20% methanol) for 1 h at 50 mA and 50 V. Recombinant H1C peptide was detected using rabbit anti-hexahistidine antibody (Santa Cruz Biotechnology) and alkaline phosphatase-conjugated anti-rabbit IgG (Sigma) diluted in antibody buffer (0.5%, v/v) Tween 20, pH 7.4. Tris-buffered saline with 1% (w/v) nonfat dry milk) with the ratio of 1:2000. The membrane was then washed and developed colorimetrically with FAST Red TR/Naphtol AS-MX (Sigma). The membrane was scanned and its image was analyzed using Gel-Pro analyzer software (Media Cybernetics).

DNA-Binding-Activity Assay. DNA-binding activity was determined with electrophoretic DNA-retardation and dot-blot assays. The recombinant H1C peptide (200 µg/mL) was mixed with the plasmid pTrcHis B (37 µg/mL; Invitrogen) at ratios (w/w) from 0.25:1 to 4:1 and incubated for 30 min at room temperature. The samples were then separated by electrophoresis at 100 V on 1% (w/v) agarose gels containing ethidium bromide, and band retardation was assessed by UV transillumination. For direct assay of DNA-binding activity, we used the dot-blot method with fluorescence-labeled DNA. Approximately 14 µg of H1C peptide and BSA (as a negative control) were blotted.
expression levels of recombinant H1C peptide were lower when recombinant goldfish H1C peptide comprising the 61 C-terminal amino acids of histone H1 (183 nucleotides) (Table 1). The expression levels of recombinant H1C peptide were lower when other E. coli cells such as BL21 (DE3) were used (data not shown). We surmised that this might be due to different codon preferences between goldfish and E. coli because C. auratus H1C has many rare codons that can create difficulties when expressed in E. coli. Thus, we used E. coli Rosetta (DE3), which is a BL21-derivative strain designed to increase expression of eukaryotic proteins that contain codons rarely used in E. coli. This strain contains a compatible chloramphenicol-resistant plasmid that provides tRNAs for the codons that are rare in E. coli (namely, AUA, AGG, AGA, CUA, CCC, and GGA). Thus, the Rosetta strain can provide “universal” translation which might otherwise be limited by the codon usage of E. coli. As expected, the recombinant H1C peptide was successfully expressed (at a concentration of ~17 μg/mL medium and comprised ~7.6% of the total cellular proteins) in E. coli Rosetta (DE3) (Figure 1, lane W). Interestingly, recombinant H1C was mainly expressed in a soluble form (Figure 1, lane S). The recombinant H1C peptide was fused with a His 6- affinity tag at the C-terminus, so the peptide was purified using IMAC under denaturing conditions to purify both the soluble and insoluble fractions (Figure 2). The recombinant H1C peptide was successfully purified with a yield of ~6.6 μg/mL (~40% of the total amount expressed) and a purity of ~98%. The apparent molecular mass of H1C on a Tricine SDS-PAGE gel was greater

Figure 2. (A) Coomassie-blue-stained SDS-PAGE and (B) Western blot analyses for affinity purification of the recombinant H1C peptide. Lanes: M, prestained protein molecular weight marker; CL, cell-lysate sample; FT, flow-through sample; W1, first-washing sample; W2, second-washing sample; E, affinity-purified fusion protein sample. 15% (w/v) gels were used for analyses.

Results and Discussion

Expression and Purification of Recombinant H1C Peptide.
Recombinant E. coli Rosetta (DE3) cells containing the pET-H1C plasmid were treated with IPTG for induction of the recombinant goldfish H1C peptide comprising the 61 C-terminal amino acids of histone H1 (183 nucleotides) (Table 1). The expression levels of recombinant H1C peptide were lower when other E. coli cells such as BL21 (DE3) were used (data not shown). We surmised that this might be due to different codon preferences between goldfish and E. coli because C. auratus H1C has many rare codons that can create difficulties when expressed in E. coli. Thus, we used E. coli Rosetta (DE3), which is a BL21-derivative strain designed to increase expression of eukaryotic proteins that contain codons rarely used in E. coli. This strain contains a compatible chloramphenicol-resistant plasmid that provides tRNAs for the codons that are rare in E. coli (namely, AUA, AGG, AGA, CUA, CCC, and GGA). Thus, the Rosetta strain can provide “universal” translation which might otherwise be limited by the codon usage of E. coli. As expected, the recombinant H1C peptide was successfully expressed (at a concentration of ~17 μg/mL medium and comprised ~7.6% of the total cellular proteins) in E. coli Rosetta (DE3) (Figure 1, lane W). Interestingly, recombinant H1C was mainly expressed in a soluble form (Figure 1, lane S). The recombinant H1C peptide was fused with a His 6- affinity tag at the C-terminus, so the peptide was purified using IMAC under denaturing conditions to purify both the soluble and insoluble fractions (Figure 2). The recombinant H1C peptide was successfully purified with a yield of ~6.6 μg/mL (~40% of the total amount expressed) and a purity of ~98%. The apparent molecular mass of H1C on a Tricine SDS-PAGE gel was greater

Figure 3. (A) Dot-blot assay to determine DNA-binding activity of recombinant H1C peptide. Blotted samples were incubated with SybrGreen-labeled pTrcHis B for 30 min at room temperature. (B) DNA-retardation assay to determine DNA-binding activity of recombinant H1C peptide. Lane M, DNA size marker. Each lane in B represents each H1C:DNA ratio (w/w). The H1C and pTrcHis B mixture was incubated for 30 min at room temperature and then loaded on 1% (w/v) agarose gel.
than the predicted molecular mass (~14 kDa versus 7.3 kDa) from the amino acid sequence (68 amino acids including His 6 tag). This is due to its high pI value (10.68) due to the high lysine content (37% of all amino acids), which is consistent with the results for other proteins with high pI values (i.e., basic proteins) (10, 18, 19). Proteins with higher pI values tend to bind more SDS molecules, leading to increased apparent molecular masses. We confirmed its correct molecular mass using matrix-assisted laser desorption ionization mass spectrometry with time-of-flight (MALDI-TOF MS) analysis of the purified H1C (data not shown).

DNA Binding of Recombinant H1C Peptide. We performed dot-blot assays using fluorescence-labeled plasmid DNA to directly measure DNA-binding ability of the recombinant H1C peptide. We found that the recombinant H1C peptide showed bright green fluorescence, whereas the control protein BSA did not show fluorescence (Figure 3A). Therefore, this assay clearly demonstrated that the recombinant goldfish H1C peptide is capable of binding DNA molecules in vitro. The recombinant H1C peptide was also assessed for its ability to form DNA-protein complexes using a gel-retardation assay. The electrophoretic mobility of the DNA in a 1% agarose gel was retarded by the presence of the H1C peptide (Figure 3B). Retardation was greater with increased amounts of the H1C peptide, and the H1C-plasmid DNA complex was completely retarded and did not migrate on an agarose gel with a H1C:DNA ratio of 2:1 (w/w). Thus, we concluded that the minimum ratio for complete binding of recombinant H1C peptide with plasmid DNA is about 2:1 (w/w).

Transfection with Recombinant H1C Peptide. As a potential protein-based system, various previous studies have demonstrated that H1-type histones are effective mediators for transfection (7, 9, 10, 20, 21). Positively charged histone H1 can tightly adhere to the cell membrane of living cells (22, 23) and has been shown to mediate efficient gene transfer in the presence of CaCl 2 and/or chloroquine (9, 10, 20). This efficient gene transfection is thought to occur through DNA condensation, DNase protection, and/or mediation of nuclear import (7, 10, 24). Of the three domains of the histone H1 molecule, the basic C-terminal domain has been shown to be largely responsible for the ability of histone H1 to bind chromatin and mediate condensation (14—16). However, a few studies have shown that the use of histone H1 C-terminal fragments can affect gene-transfer efficiency. For example, the C-terminal domain of human histone H16 was fused to the SV40 large T-antigen nuclear-localization signal and used to transfected mammalian cells in the presence of chloroquine (7). A recombinant long C-terminal peptide of ~140 amino acids from human histone H1 could also be used for efficient delivery of DNA and RNA into transfected mammalian cells with very low toxicity (10).

Here, we expressed and purified the recombinant goldfish histone H1-derived short peptide, H1C comprising the C-terminal 61 amino acids, and showed that it has good DNA-binding ability. On the basis of its confirmed DNA-binding ability, we investigated whether the recombinant H1C peptide can deliver DNA into human 293T cells. As a target gene, we used lacZ that encodes β-galactosidase (Figure 4A). As measured by β-galactosidase expression levels, transfection efficiencies using several combinations of recombinant H1C peptide were similar to or higher than with the widely used lipofectamine-mediated method. Note that we used lipofectamine 2000 which is developed to be used with serum addition for better transfection. In particular, the H1C:DNA ratio of 8:1 (w/w) showed the highest (~1.5-fold) transfection efficiency. We confirmed the potency of recombinant H1C peptide using another reporter gene, egfp, which encodes EGFP (Figure 4B,C). When we used a ratio of 8:1 (w/w) for H1C-mediated transfection, the EGFP expression was higher (~1.7-fold) than with the lipofectamine 2000 method. Therefore, we confirmed that the short C-terminal histone H1 peptide of 61 amino acids can be successfully used for gene delivery. Interestingly, when transfection was performed in the absence of calcium ions, we found that H1C-mediated transfection did not occur (Figure 4B), consistent with previous reports on the role of calcium ions in histone H1-mediated transfection (20, 25). It has been suggested that, although uptake of transfection complexes is a Ca 2+-independent process, calcium ions are required to release the transfection complexes from the endosome/lysosome compartments (25) and this can be inhibited by serum in the medium.
The recombinant histone H1 C-terminal peptide showed good DNA-binding ability and was found to have similar or higher (at a certain mixed ratio) transfection efficiency in human 293T and mouse NIH/3T3 cells than the widely used lipofectamine method. Taken together, these findings indicate that this novel goldfish-derived histone H1 C-terminal short peptide could be used as a potential peptide-based mediator for efficient gene delivery.

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References and Notes

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Conclusion

Here, we successfully expressed and purified the recombinant C. auratus histone H1-derived peptide comprising the 61 C-terminal amino acids in an E. coli expression system. The peptide was expressed mainly in a soluble form through the use of a His6-tag fusion strategy and supplying tRNAs for rare codons. The recombinant histone H1 C-terminal peptide showed good DNA-binding ability and was found to have similar or higher (at a certain mixed ratio) transfection efficiency in human 293T and mouse NIH/3T3 cells than the widely used lipofectamine method. Taken together, these findings indicate that this novel goldfish-derived histone H1 C-terminal short peptide could be used as a potential peptide-based mediator for efficient gene delivery.


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