0273-2289/1559-0291 (Online)/06/133/0149/$30.00

Expression, Refolding, and Characterization of GFE Peptide-Fused Human Interferon-α2a in *Escherichia coli*

Zhen Yan,[1,2] Li Lu,[3] Jihong Shi,[1,2] Chunjie Bao,[1,2] Wei Han,[1,2] Yongjie Wu,[3] and Yingqi Zhang*,[1,2]

[1]Biotechnology Center of Fourth Military Medical University, Chang Le Xi Lu #17, Xi'an 710032, Shaanxi, People's Republic of China, E-mail: zhangyqh@fmmu.edu.cn; [2]State Key Laboratory of Cancer Biology, Chang Le Xi Lu #17, Xi'an 710032, Shaanxi, People's Republic of China; and [3]College of Pharmaceutical Science of Lanzhou University, Key Laboratory of Preclinical Study for New Traditional Chinese Medicine of Gansu Province, Lanzhou University, Lanzhou 730000, Gansu, People's Republic of China

Received July 12, 2005; Accepted October 30, 2005

Abstract

Interferon-α2a (IFN-α2a) has been used for the treatment of various viral infections and cancers for many years. However some untolerable side effects have limited its application in some aspects. To evaluate whether or not an oligopeptide containing GFE motif can home human IFN-α2a to specific tissues, a fusion gene was constructed by fusing the coding sequence of GFE peptide (CGFECVRQCPERC), which was screened from phage display peptide library, to the 3′ end of human IFN-α2a gene by recombinant DNA technique. Fusion protein rhIFN-α2a-GFE was expressed in *Escherichia coli* as inclusion bodies using a T7 RNA polymerase expression system, pET-22b, refolded through dialysis and purified to homogeneity to >95% of purity by affinity chromatography. Characterization by sodium dodecyl sulfate polyacrylamide gel electrophoresis and immunoblotting demonstrated the authenticity of the fusion protein. Purified rhIFN-α2a-GFE was found to be functionally active in terms of its antiviral activity for about 2.5×10^8 IU/mg in vitro. Yields of the purified fusion protein were about 200 mg/L of culture medium. Tissue distribution assay in mouse showed that at 30 min IFN-α2a could be enriched sevenfold higher in lung in the targeted IFN group of mice

*Author to whom all correspondence and reprint requests should be addressed.

than in the standard IFN group of mice, and last for a long time. At 1 h, IFN-α2a in the targeted IFN group was still 4.02-fold higher than that in the standard group. This confirmed that GFE peptide has the ability to selectively deliver its fusion partner IFN-α2a to lungs. The results also showed that the IFN-α2a-GFE could be specifically enriched in kidney and liver. Its distribution in kidney was concordant with the finding of GFE receptor, MDP, in kidney. However, the IFN-α2a-GFE in liver may imply some significance in pharmacology and toxicology.

Index Entries: DNA recombination; expression; interferon-α2a; purification; target peptide; tissue distribution.

Introduction

For the past 40 yr, various forms of interferon (IFN) have been evaluated as therapy in a number of malignant and nonmalignant diseases. Since their discovery, IFNs have been divided into two major subgroups by virtue of their ability to bind to common receptor types *(1–3)*. Type I IFNs all bind to a type I IFN receptor and include IFN-α, IFN-β, IFN-ω, IFN-κ, and IFN-τ. IFN-γ is the sole type II IFN and binds to a distinct type II receptor *(4)*.

The IFNs possess a broad spectrum of activity and are involved in complex interactions *(3–5)*. They display antiviral activity *(6–8)* and impact cellular metabolism and differentiation. IFN-α has been approved for the treatment of chronic hepatitis B; chronic hepatitis C; and papilloma virus infections, which cause genital warts.

IFNs also have an antiproliferative effect on tumor cells *(9–13)*; that is, they inhibit their growth directly. At the same time, IFNs stimulate the tumor cells to change their surfaces so that they are recognized by the immune system as abnormal cells. IFNs block the growth of new blood vessels and help cut off the supply of nutrients. In oncology, IFNs are an important treatment for a number of solid tumors and hematologic malignancies. These include melanoma, renal cell carcinoma, AIDS–related Kaposi sarcoma, follicular lymphoma, hairy cell leukemia, and chronic myelogenous leukemia . Approximately 180 clinical trials utilize IFN in cancer today. Among them, IFN-α is the predominant one.

However, IFN therapy is associated with significant side effects *(6–13)* such as fever, chills, rigor, headache, myalgia, malaise, fatigue, anorexia, and neuropsychiatric symptoms, which have an impact on the patient's quality of life and the physician's ability to treat the patient optimally. To improve the efficiency of IFN treatment, we tested whether or not some tissue homing oligopeptides can target IFN-α2a to specific tissues. Here, we report the design of a lung-targeting IFN-α2a. Lung-homing oligo-peptide *(14)* (CGFECVRQCPERC, abbreviated as GFE) was fused to the C-terminal of human IFN-α2a (hIFN-α2a) by recombinant technique. The fusion artificial protein was expressed in *Escherichia coli*, purified through protein refolding and affinity chromatography, and then characterized.

Materials and Methods

Generation of Targeted IFN-α2a

GFE peptide was designed to fuse to the 3' end of human IFN-α2a gene. IFN-α2a gene was amplified by polymerase chain reaction (PCR) from human leukocyte cDNA library. Primers were synthesized (in Bioasia Co. in Shanghai) according to the human IFN-α2a gene sequence in Gene bank *(15)*. The forward primer is CCCATATGTGTGATCTGCCTCAAAC, and the backward primer is CCGGATCCTTCCTTACTTCTTAAACT. *Nde*I and *Bam*HI restriction endonuclease recognition sequences were added to the 5' and 3' end, respectively, which are underlined. The PCR was subjected to a program consisting of a DNA denaturation step at 94°C for 2 min, 30 cycles at 94°C for 40 s, 52°C for 40 s, and 72°C for 1 min based on the average melting temperature (*Tm*) of the oligonucleotides. The coding sequence of GFE peptide (CGFECVRQCPERC) was synthesized according to the codon usage in *E. coli* as follows: forward: GATCCTGCGGTTTCG AATGCGTTCGTCAGTGCCCGGAACGTTGCTAGG; backward: TCGAC CTAGCAACGTTCCGGGCACTGACGAACGCATTCGAAACCGCAG. *Bam*HI and *Sal*I restriction endonuclease recognition sequences were added to each end, respectively, and underlined. A stop codon, TAG, was used, as shown in the box. GFE peptide coding sequence was annealed at 70°C for 10 min and then cooled to 25°C gradually. Mixed and ligated with agarose gel electrophoresis purified, *Nde*I and *Bam*HI digested IFN-α2a fragment after PCR, and *Nde*I and *Sal*I digested pET-22b(+) expression plasmid vector (Novagen). After transforming *E. coli* DH5α, the recombinant colonies were first analyzed by agarose gel electrophoresis for correct DNA insert fragment identification after proper restriction enzyme digestion of recombinant plasmid DNA, and followed by DNA sequencing. The correct construct was called pET-IFN-α2a-GFE.

Fusion Protein IFN-α2a-GFE Expression in E. coli

Recombinant plasmid pET-IFN-α2a-GFE was transformed into *E. coli* BL21(DE3). The expression system BL21(DE3)(pET-IFN-α2a-GFE) was ampicillin resistant and has a lac operon in conjunction with the T7 promoter to ensure stronger control over target gene expression. Recombinant gene expression was first confirmed in a test tube in which BL21(DE3)(pET-IFN-α2a-GFE) was grown in Luria-Bertani (LB) medium containing 100 mg/L of ampicillin at 37°C. The production of recombinant protein was achieved by the addition of 0.5 m*M* isopropyl-β-D-thiogalactoside (IPTG) to the culture medium when OD_{600nm} was about 0.6. Cells were harvested 3 h later and analyzed by 6–15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Fermentation was then carried out in a BIOSTAT C-5 fermentor (working volume of 5 L) (B. Braun Biotech). The seed buildup was conducted in two stages. In the first stage, a single colony was picked up and inoculated in 5 mL of LB-amp medium and grown overnight at 37°C. The next morning

1% overnight culture was inoculated into 5 mL of fresh LB-amp medium, grown to an OD_{600nm} of 0.6, then stored at 4°C. In the second stage, a 500-mL shake flask with 150 mL of sterile seed medium (16 g/L of tryptone, 10 g/L of yeast extract, 20 g/L of glycerol, 5 g/L of NaCl, 100 mg/L of ampicillin) was inoculated with 1% first-stage production and grown at 37°C for about 8 h. The next morning, 5 L of sterile medium (5 g/L of tryptone, 5 g/L of yeast extract, 10 g/L of glycerol, 2 g/L of KH_2PO_4, 4 g/L of K_2HPO_4, 3 g/L of $MgSO_4$) in the fermentor was inoculated aseptically with 150 mL (3% [v/v]) of the second-stage seed. During fermentation, the seed was fed-batched with 1000 mL sterile medium (60 g/L of tryptone, 40 g/L of yeast extract, 400 g/L of glycerol, 5 g/L of $MgSO_4$). Antifoam was added to 0.1% (v/v). The fermentor was maintained at 37°C with an agitation rate from 250 rpm increasing to 850 rpm, an aeration rate from 2 L/min increasing to 6 L/min, and a vessel pressure of up to 0.6 bar. The %DO (percent of dissolved oxygen) within the fermentor was maintained at >20% by adjusting the aeration rate, agitation rate, and vessel pressure incrementally. The culture was induced with sterilized IPTG at a final concentration of 1.5 m*M* when the OD_{600nm} reached 7 (±1.0). The culture was prepared for harvest 4 h after IPTG induction. The cell paste was recovered by centrifuging at 3500*g* in 15 min at 4°C (Beckman J-6B). The fermentation yielded approx 200 g of wet cell paste that was divided into 10-g aliquots and stored at –70°C until proceeding to the recombinant protein purification of the process.

Refolding and Purification of rhIFN-α2a-GFE

The recombinant fusion protein rhIFN-α2a-GFE was expressed in an insoluble form as inclusion bodies. The cells (10 g) were resuspended in lysis buffer at a ratio of 1:5 (100 mmol/L of NaCl; 50 mmol/L of Tris-HCl, pH 8.0; and 1 mmol/L of EDTA) and lysed by lysozyme. Cellular lysates were centrifuged at 25,000*g* for 15 min at 4°C (Beckman J2-HS). Inclusion bodies were washed with 200 mL of washing buffer (100 mmol/L of NaCl; 50 mmol/L of Tris-HCl, pH 8.0; 1 mmol/L of EDTA; 2 *M* urea; 2% Triton X-100; and 1 m*M* β-mercaptoethanol). After centrifugation as just described, the inclusion bodies were dissolved in 250 mL of 7 *M* guanidine HCl then dialyzed against 10 L of phosphate-buffered saline (PBS), pH 7.2.

After centrifuging at 16,000*g* for 10 min at 4°C, the supernatant was applied to a column of antihuman IFN-α2a monoclonal antibody (MAb)–conjugated Sepharose 4B (Immunology Department, Fourth Military Medical University [FMMU]). Contaminants were washed away with 10 vol of PBS, pH 7.2, and 1 vol of 0.5% Tween-20 sequentially. The fusion protein rhIFN-α2a-GFE was then eluted with a buffer containing 0.2 *M* NaCl and 0.1 *M* glycine, pH 2.5. The final products were stored at –20°C.

Characterization of rhIFN-α2a-GFE

Total Protein Analysis

Total protein concentrations of samples were determined using Lowry's method *(15a)*.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

Samples taken at various stages of purification were analyzed by 6–15% SDS-PAGE. Samples were dissolved in SDS-PAGE loading buffer containing 200 m*M* dithiothreitol (DTT) and 1% β-mercaptoethanol in reducing conditions or without reducing agent in nonreducing conditions. Gels were electrophoresed at 200 V (constant voltage) for 30 min and stained with Coomassie brilliant blue R250.

Immunoblotting

Proteins were separated on 6–15% SDS-PAGE and electrophoretically transferred onto nitrocellulose membrane (Schleicher & Schuell) in a Trans-Blot apparatus (Bio-Rad) with transfer buffer (192 m*M* glycine; 25 m*M* Tris base; and 20% methanol, pH 8.3). The membrane was blocked with 1% bovine serum albumin (BSA) in Tris-buffered saline (20 m*M* Tris, pH 7.6; 140 m*M* NaCl) containing 0.1% Tween-20 (TBST) for 1 h, followed by incubation with a 1:1000 dilution of monoclonal antihuman IFN-α2a antibodies in TBST for 1 h. The membrane was then washed three times with TBST in 15 min and incubated with a 1:5000 dilution of goat antimouse alkaline-phosphate (AP)-linked secondary antibody for 1 h. After washing thoroughly, protein bands were stained with Western blue stabilized substrate.

Size-Exclusion Chromatography-High-Performance Liquid Chromatography

Size-exclusion chromatography (SEC) was performed in PBS with 0.1 *M* NaCl, pH 6.8, using a Beckman's high-performance liquid chromatography (HPLC) system. Five micrograms of sample in PBS containing 200 m*M* DTT and 1% β-mercaptoethanol in reducing conditions or without reducing agent in nonreducing conditions was injected onto a 7.5 × 300 mm G2000SW column (Tosoh) at a flow rate of 0.5 mL/min. Peaks were detected by monitoring at a wavelength of 280 nm. The purity of rIFN-α2a-GFE was calculated as a percentage of the total peak area detected.

Antiviral Properties of rhIFN-α2a-GFE Fusion Protein

Antiviral specific activity of the fusion protein was determined on human cells by using cytopathic effect (CPE) inhibition assays *(16,17)*. Briefly, 100 μL of WISH (human amniotic cell line, 3×10^5 cells/mL) cultured in MEM medium was seeded in a 96-well microplate. One hundred microliters of twofold serial diluted IFN preparations (10^3 IU/mL) was added to each well. After incubating for 24 h at 37°C with 5% CO_2, the cells were infected with vesicular stomatitis virus (Indiana strain) ($100TCID_{50}$), followed by an additional 24-h incubation. Every sample was done in triplicate. The CPE was checked by OD_{570nm} absorbant after staining with crystal violet on virus control, cell control, and cells that received State Food and Drug Administration (SFDA) in China standard IFN. (The highest dilution giving 50% reduction of the viral plaques was considered the end point.) The results were calculated by a computer program and expressed as international units per milliliter.

Tissue and Organ Distribution Assay in Animals

Male and female Kunming mice, with an average body wt of 20 g, from the Experimental Animal Research Center of FMMU in China were used and divided into three groups. Each group contained six mice. Mice in the standard IFN group and the targeted IFN group were challenged by iv injection of equimolar rhIFN-α2a or rhIFN-α2a-GFE, 4.5 n*M*/kg, with an average volume of 0.2 mL/10 g, respectively. The control group was challenged by an equal volume of physiologic saline intravenously. Three animals in each group were sacrificed under ether anesthesia at 30 min or 1 h after injection. Blood was taken from orbital after eyeball extirpating and sera were prepared by centrifugation. Heart, liver, spleen, lung, and kidney were separated and homogenized in 3 mL of PBS, pH 7.4, with 1% BSA in every gram of tissue. After centrifugation, the supernatant was diluted fivefold with dilution buffer from an hIFN-α enzyme-linked immunosorbent assay (ELISA) kit (Bender Medsystem). Then ELISA was carried out and IFN-α concentrations were determined according to the manuscript procedure in the Bender Medsystem ELISA kit.

Results and Discussion

Amplification of hIFN-α2a Gene

hIFN-α2a gene was amplified from human leukocyte cDNA library by PCR through two oligonucleotides. One percent agarose gel electrophoresis revealed the presence of a major DNA band of approx 500 bp, which corresponds to the IFN-α2a gene (Fig. 1). The presence of a smaller blur band in this gel could be attributed to a primer dimer formation.

Generation of hIFN-α2a-GFE Fusion Gene

GFE peptide is a lung-homing peptide, composed of 13 amino acid residues, which was identified by in vivo phage display *(14)*. Study showed that this peptide could specifically bind to membrane dipeptidase (MDP) in lung, a cell-surface zinc metalloprotease involved in the metabolism of glutathione, leukotriene D_4, and certain β-lactam antibiotics *(18)*. To investigate whether or not the GFE peptide can target human IFN-α2a to lung tissue when used systemically, we decided to construct and express a fusion gene in which the GFE peptide coding sequence was fused to the 3′ end of hIFN-α2a gene.

The amplified hIFN-α2a gene was digested with *Nde*I and *Bam*HI and gel purified together with synthesized and annealed GFE peptide coding sequence and cloned into the pET-22b(+) vector at the *Nde*I/*Sal*I restriction sites. Their sequences were conformed by restriction enzyme digestion (Fig. 2) and DNA sequencing (data not shown). The correct construct was designated as pET-IFN-α2a-GFE.

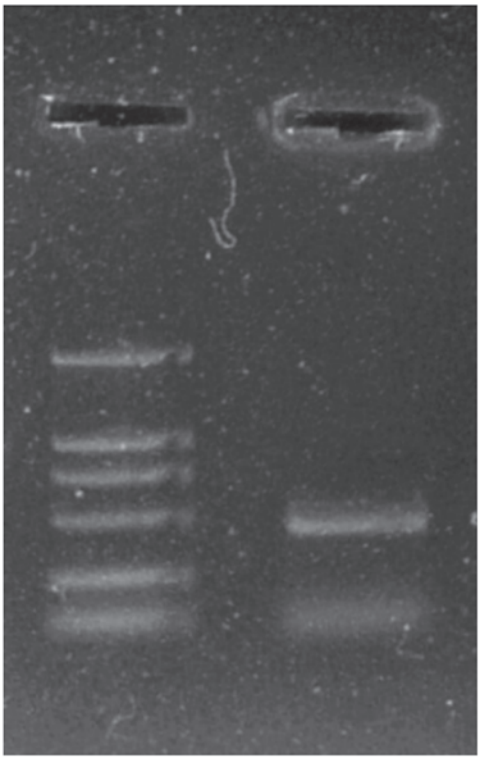

Fig. 1. Analysis of PCR products from amplification of hIFN-α2a gene on 1% agarose gel electrophoresis: lane 1, from left to right, DNA molecular mass marker DL2000, and from top to bottom, 2000, 1000, 750, 500, 250, and 100 bp); lane 2, PCR products.

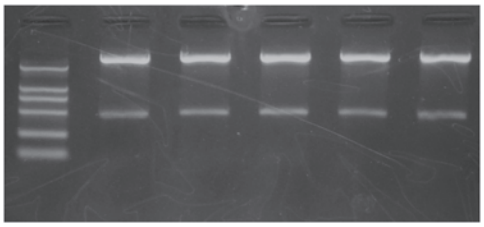

Fig. 2. Agarose gel electrophoresis of recombinant plasmids pET- IFN-α2a-GFE after digestion with *Nde*I and *Sal*I: lane 1, DNA molecular mass marker DL2000; lanes 2–6, plasmids no. 1–5 after digestion with *Nde*I and *Sal*I.

Expression of rhIFN-α2a-GFE Fusion Protein

The recombinant plasmid pET-IFN-α2a-GFE was introduced into *E. coli* BL21 (DE3), and the expression of the recombinant hIFN-α2a-GFE fusion protein (rhIFN-α2a-GFE) was induced by the addition of IPTG in the culture medium. After culture in the presence of IPTG for about 4 h, a novel protein was induced in *E. coli* transformed with pET-IFN-α2a-GFE, but not

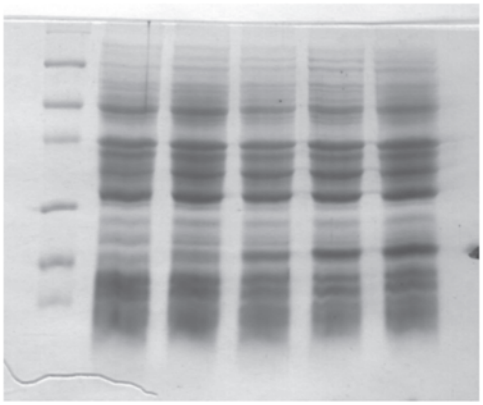

Fig. 3. Analysis of recombinant protein expression (IPTG induction) after fermentation on 15% SDS-PAGE: lane 1, protein molecular mass standards (from top to bottom: β-galactosidase, 116 kDa; BSA, 66 kDa; ovalbumin, 45 kDa; lactate dehydrogenase, 35 kDa; restriction endonuclease Bsp981, 25 kDa; β-lactoglobulin, 18.4 kDa; lysozyme, 14.4 kDa); lane 2, cell lysate before induction; lane 3, cell lysate 1 h after induction (5.167% rhIFN-α2a-GFE by SDS-PAGE densitometry); lane 4, cell lysate 2 h after induction (7.64% rhIFN-α2a-GFE by SDS-PAGE densitometry); lane 5, cell lysate 3 h after induction (9.88% rhIFN-α2a-GFE by SDS-PAGE densitometry); lane 6, cell lysate 4 h after induction (10.988% rhIFN-α2a-GFE by SDSPAGE densitometry).

pET-22b (+). The molecular weight of the induced novel protein was about 21 kDa on SDS-PAGE, consistent with the calculated molecular mass of the rhIFN-α2a-GFE fusion protein (21.05 kDa), suggesting that rhIFN-α2a-GFE was expressed successfully in *E. coli.*

Initial studies indicated that there was some colony-to-colony variability in the productivity of rhIFN-α2a-GFE fusion protein. A high-expression clone was identified by screening a large number of subclones and used for all studies described throughout the article.

To scale up the production of rhIFN-α2a-GFE, 5-L-scale fermentation was performed. During fermentation, the pH of the medium decreased gradually, usually approaching 6.4 at the end of fermentation; however, the pH was only monitored, not controlled, during fermentation. Figure 3 shows the increased rhIFN-α2a-GFE expression up to 4 h after IPTG induction. The average production runs yielded 200 g of cell paste in 5 L of culture.

Refolding and Purification of Fusion Protein

The fusion protein rhIFN-α2a-GFE was expressed as insoluble form, as shown in Fig. 4. After cell lysis by lysozyme, the inclusion bodies were isolated, washed with urea, solubilized in guanidine HCl, dialyzed against PBS (pH 7.2), and purified in one step by MAb affinity chromatography (Fig. 4). For 10 g of wet cell pellet, about 50 mg of rhIFN-α2a-GFE fusion protein could be recovered. Table 1 summarizes the purification process.

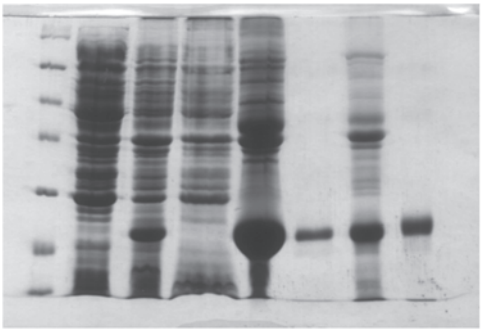

Fig. 4. Analysis of protein purification on 15% SDS-PAGE: lane 1, protein molecular mass standard; lane 2, supernatant of cell lysate; lane 3, pellet of cell lysate; lane 4, washing supernatant of inclusion body; lane 5, washing pellet of inclusion body; lane 6, precipitation after dialysis; lane 7, refolded rhIFN-α2a-GFE after dialysis; lane 8, elution of recombinant protein rhIFN-α2a-GFE from affinity chromatography.

Table 1
Process Summary and Yields

Sample	Concentration (mg/mL)	Volume (mL)	Total protein (g)	Recovery (%)	Yield (%)
Cell paste			10		
Washed/pelleted inclusion bodies			3	30	30
Solubilized inclusion bodies	12	250	3		
Refold/after dialysis	0.44	270	0.119	4	1.19
Affinity chromatography–purified rhIFN-α2a-GFE	1.05	50	0.052	44	0.52

Thus, the yield of purified fusion protein rhIFN-α2a-GFE could achieve 200 mg/L of culture. This result has met the high level of reported recombinant IFN production *(19–21)*. The process could be extended to other recombinant proteins of interest.

Purity, Binding Ability, and Antiviral Activity Analysis of Final Product

The final purified product was evaluated to ensure its quality. SDS-PAGE Coomassie brilliant blue-stained gels run under reducing conditions showed a single band at 21 kDa, which was consistent with the predicted molecular mass of hIFN-α2a-GFE (21.05 kDa). Nonreducing SDS-PAGE gels showed three bands at a molecular mass of about 20, 40, and 60 kDa, respectively (Fig. 5A). To confirm that these higher molecular mass bands were formed with homodimer or homotrimer of recombinant protein, immunoblotting was carried out. The results showed that both the single band on reducing conditions and the three bands on nonreducing conditions could bind to antihuman IFN-α2a MAb specifically (Fig. 5B), which

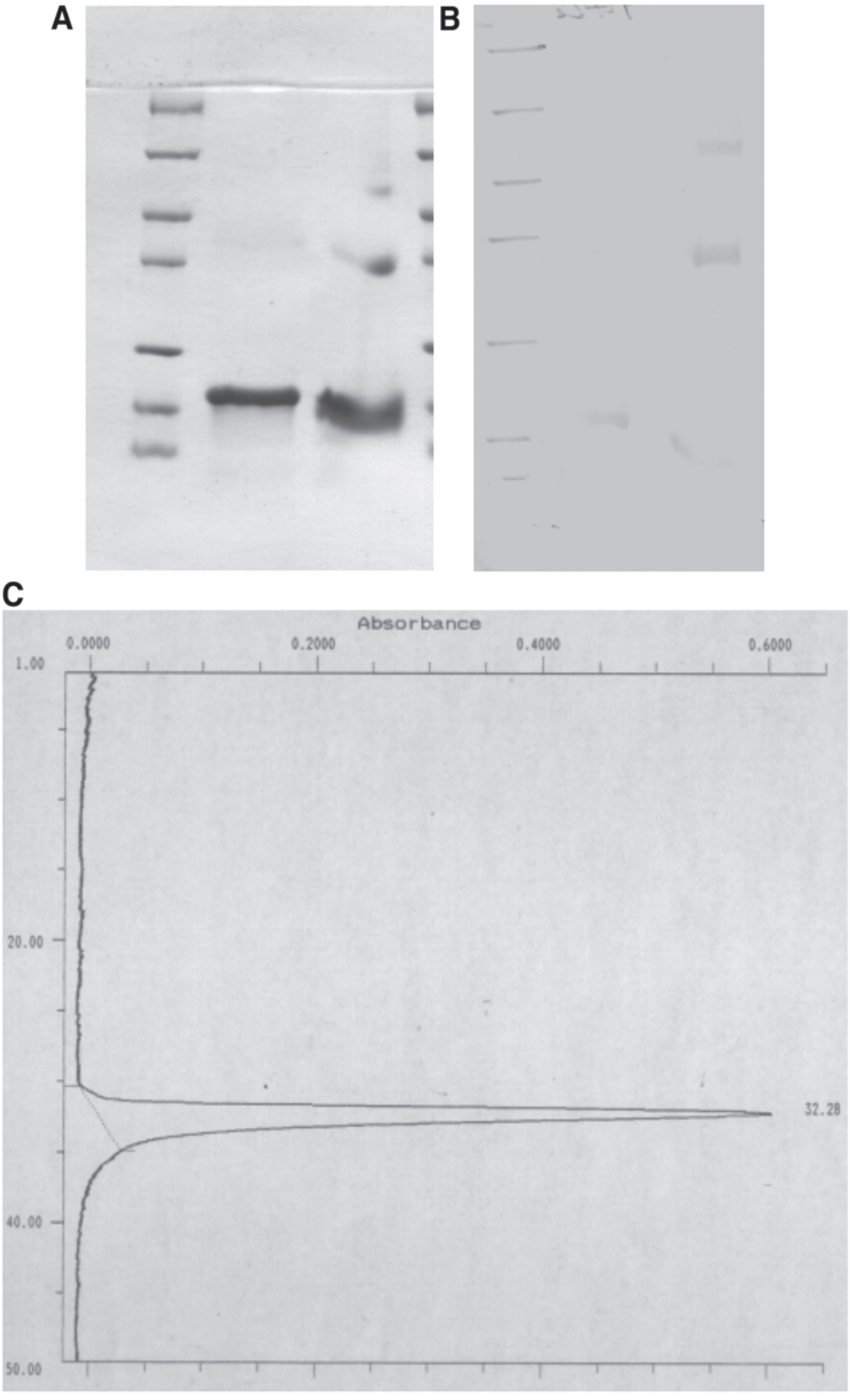
A
B
C
Absorbance
0.0000
0.2000
0.4000
0.6000
1.00
20.00
40.00
50.00
32.28

Fig. 5.

implies that the *E. coli*-expressed GFE-fused hIFN-α2a may exist as a mixture of monomer, dimer, and trimer. SEC-HPLC analysis further confirmed the result: in PBS solution, the final purified product could be divided into three peaks (data not shown); however, in reducing solution, the purified protein existed in only one peak, and the area of the peak was >95% (Fig. 5C). When CPE inhibition assay was employed to estimate the purified protein's antiviral activity, the results showed an average specific activity of 2.5×10^8 IU/mg, a little bit lower than the Chinese SFDA standard hIFN-α2a (~5 H 10^8 IU/mg). From these findings, we can see that the artificial fusion protein rhIFN-α2a-GFE retained the structure, binding, and antiviral ability of natural hIFN-α2a well. The dimer and trimer of rhIFN-α2a-GFE had no significant effect on IFN-α2a's antiviral activity.

Tissue and Organ Distribution of IFN-α2a-GFE Fusion Protein

Distribution experiments were carried out in mice as described in Materials and Methods. ELISA assay showed that there was no significant difference in plasma IFN-α2a concentration between the standard IFN-α2a group and the targeted IFN-α2a group at 30 min and 1 h after equal molar intravenous injection of rhIFN-α2a or rhIFN-α2a-GFE. The percentages of IFN-α2a concentrations in different tissues at different times based on plasma concentrations were calculated and are summarized in Table 2. At 30 min, IFN-α2a could be enriched sevenfold higher in lung in the targeted group of mice than in the standard IFN-α2a group of mice, and last for a long time. At 1 h, the IFN-α2a in the targeted group was still 4.02-fold higher than that of the standard group. This confirmed that GFE peptide is a lung-homing peptide, and that it has the ability to selectively deliver its fusion partner IFN-α2a to lungs. However, the original research reported that GFE phage had more than 35-fold selectivity in lung homing relative to a control phage *(14)*. The lowered lung-homing ability may be caused by a conformation obstacle after protein fusion.

The results also showed that IFN-α2a-GFE could be specifically enriched in kidney and liver. In kidney, IFN-α2a concentrations were 7.79- and 5.4-fold relative to those of the standard IFN-α2a group at 30 min and 1 h, respectively. In liver, the differences were 4.52- and 13.96–fold, respectively. The statistical diversities are significant ($p < 0.01$).

Fig. 5. *(previous page)* Purity of the final purified rhIFN-α2a-GFE. **(A)** Coomassie SDS-PAGE gel: lane 1, molecular mass standards; lane 2, 10 μg of purified rhIFN-α2a-GFE under reducing conditions; lane 3, 10 μg of purified rhIFN-α2a-GFE under nonreducing conditions. **(B)** Immunoblotting analysis of rhIFN-α2a-GFE. Proteins blotted onto nitrocellulose membranes were probed with antihuman IFN-α MAb, followed by antimouse AP-linked secondary antibody incubation. Bands were revealed with a BCIP/NBT system. Lane 1, molecular mass standards stained with light green; lane 2, rhIFN-α2a-GFE under reducing conditions; lane 3, rhIFN-α2a-GFE under nonreducing conditions. **(C)** SEC-HPLC: 5 μg of purified rhIFN-α2a-GFE under reducing conditions and analyzed on G2000SW column; detection was at OD_{280nm} and peak purity was >95%.

Table 2
Tissues and Organs Distribution of rhIFN-α2a and rhIFN-α2a-GFE in Mice

	rhIFN-α2a-GFE				rhIFN-α2a			
Tissue	30 min (pg/mL)	Plasma concentration (%)	1 h (pg/mL)	Plasma concentration (%)	30 min (pg/mL)	Plasma concentration (%)	1 h (pg/mL)	Plasma concentration (%)
Plasma	2418.06 ± 622.46	100	1420.32 ± 341.42	100	2641.72 ± 476.69	100	1125.03 ± 180.21	100
Heart	563.75 ± 306.47	23.31	234.43 ± 93.41	16.51	418.06 ± 93.80	15.83	143.68 ± 35.72	12.77
Liver	420.35 ± 199.27[a]	17.38	205.74 ± 46.87[b]	14.49	93.00 ± 18.65	3.52	14.74 ± 8.85	1.3
Spleen	305.65 ± 142.95	12.64	147.40 ± 33.74	10.38	155.45 ± 58.27	5.88	89.33 ± 23.97	7.94
Lung	2998.8 ± 695.0[b]	124.03	1366.4 ± 328.0[a]	96.20	430.14 ± 120.80	16.28	339.52 ± 253.50	30.2
Kidney	4098.10 ± 1017.08[b]	169.48	1653.48 ± 403.10[b]	116.41	525.84 ± 134.59	19.91	306.13 ± 101.15	27.21

[a] $p < 0.5$ vs rhIFN-α2a.
[b] $p < 0.01$ vs rhIFN-α2a.

MDP is found to be the receptor of GFE peptide *(18,22)*, which is a plasma membrane glycosylphosphatidylinositol-anchored glycoprotein, with a molecular mass of 48–59 kDa. MDP is found to be a zinc metalloprotease involved mainly in the metabolism of glutathione. It is expressed mainly in lung and kidney *(22)*. In the lung, MDP expression has been reported on the epithelial cells of the conducting airways, epithelial cells of the alveolar ducts, capillaries, and basement membrane of alveoli and terminal bronchioles. MDP expression has also been observed on the endothelial cells of submucosal microvessels in the human trachea *(23)*. The level of MDP activity was shown to be highest in the lung *(24,25)*. In the kidney, MDP expression is concentrated in the brush-border region of the proximal tubules *(26)*. A study on mice reported that four distinct MDP mRNAs are present and differentially expressed in several organs *(27)*. The most concentrated organs are small intestine, kidney, and lung. Much lower levels were seen in heart and skeletal muscle. No MDP was detected in both adult and fetal liver. The results of our experiment coincide with MDP expression in lung and kidney. These findings suggest some potential application of the fusion protein in lung and kidney. However, the enrichment of GFE-fused IFN-α2a in liver may imply some pharmacologic and toxicologic significance. Further study is needed to elucidate whether MDP or its isoforms exist in liver, or whether some other kind of GFE receptor exists.

Conclusion

IFN-α2a is a widely used therapeutic cytokine for some tumors and viral infection diseases. However, some severe side effects limit its application. To develop targeting IFN, an hIFN-α2a fusion protein was constructed by employing a lung-homing peptide GFE to the C-terminal of hIFN-α2a. By using gene synthesis, recombinant cloning techniques, and a high-yield one-step purification procedure, we were able to produce a biologically active rhIFN-α2a-GFE fusion protein. The fusion protein could be enriched specifically in lung and kidney, suggesting a potential application of GFE peptide to deliver its fusion partner IFN-α2a to lung and kidney.

Acknowledgments

We are grateful to Prof. Han Hua (Department of Genetic and Developmental Biology, FMMU) for critically reading the manuscript. This work was supported by the program Changjiang Scholars and Innovative Research Team in University (PCSIRT).

References

1. Isaacs, A. and Lindenmann, J. (1957), *Proc. R. Soc. London Ser. B* **147,** 258–267.
2. Aguet, M., Grobke, M., and Dreiding, P. (1984), *Virology* **132,** 211–216
3. Meager, A. (2002), *J. Immunol. Methods* **261,** 21–36.
4. Pfeffer, L. M., Dinarello, C. A., Herberman, R. B., Williams, B. R., Borden, E. C., Bordens, R., Walter, M. R., et al. (1998), *Cancer Res.* **58,** 2489–2499.

5. Sen, G. C. and Lengyel, P. (1992), *J. Biol. Chem.* **267,** 5017–5020.
6. Samuel, C. E. (2001), *Clin. Microbiol. Rev.* **14,** 778–809.
7. Lauer, G. M. and Walker, B. D. (2001), *N. Engl. J. Med*. **345,** 41–52.
8. Samuel, C. E. (1991), *Virology* **18,** 31–11.
9. Jonasch, E. and Haluska, F. G. (2001), *Oncologist* **6,** 34–55.
10. Vilcek, J. and Sen, G. C. (1996), in *Fundamental Virology,* 3rd ed (Fields, B. N., Knipe, D. M., and Howley, P. M., eds.), Lippincot-Raven, Philadelphia, pp. 341–365.
11. Motzer, R. J., Bacik, J., Murphy, B. A., Russo, P., and Mazumdar, M. (2002), *J. Clin. Oncol.* **20,** 289–296.
12. Mahon, F. X., Delbrel, X., Cony-Makhoul, P., Faberes, C., Boiron, J. M., Barthe, C., Bilhou-Nabera, C., et al. (2002), *J. Clin. Oncol.* **20,** 214–220.
13. Stark, J. J., Dillman, R. O., Schulof, R., Wiemann, M. C., Barth, N. M., Honeycutt, P. J., and Soori, G. (1998), *Cancer* **82,** 1677–1681.
14. Rajotte, D., Arap, W., Hagedorn, M., Koivunen, E., Pasqualini, R., and Ruoslahti, E. (1998), *J. Clin. Invest.* **102,** 430–437.
15. Weissmann, C. and Weber, H. (1986), *Prog. Nucleic Acid Res. Mol. Biol.* **33,** 251–300.
15a. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem.* **193,** 265–275.
16. Familletti, P. C., Rubinstein, S., and Pestka, S. (1981), *Methods Enzymol.* **78,** 387–394.
17. Standardization Committee of Department of Health in China. (2000), in *Chinese Regulation for Biological Products* (Pan, Z. A., Zhang, X. H., and Duan, Z. B., eds.), Chinese Chemical Industry Press, Beijing, China, pp. 373–375.
18. Rajotte, D. and Ruoslahti, E. (1999), *J. Biol. Chem.* **274,** 11,593–11,598.
19. Thatcher, D. R. and Panayotatos, N. (1996), *Methods Enzymol.* **119,** 166–177.
20. Swaminathan, S. and Khanna, N. (1999), *Protein Express. Purif.* **15,** 236–242.
21. Neves, F. O., Ho, P. L., Raw, I., Pereira, C. A., Moreira, C., and Nascimento, A. L. (2004), *Protein Express. Purif.* **35,** 353–359.
22. Keynan, S., Hooper, N. M., and Turner, A. J. (1996), in *Zinc Metalloproteases in Health and Disease,* Hooper, N. M., ed., Taylor & Francis, London, pp. 285–309.
23. Yamaya, M., Sekizawa, K., Terajima, M., Okinaga, S., Ohrui, T., and Sasaki, H. (1998), *Respir. Physiol.* **111,** 101–109.
24. Hirota, T., Nishikawa, Y., Tanaka, M., Igarashi, T., and Kitagawa, H. (1986), *Eur. J. Biochem.* **160,** 521–525.
25. Pytela, R., Pierschbacher, M. D., and Ruoslahti, E. (1985), *Cell* **40,** 191–198.
26. Habib, G. M., Shi, Z. Z., Cuevas, A. A., Guo, Q., Matzuk, M. M., and Lieberman, M. W. (1998), *Proc. Natl. Acad. Sci. USA* **95,** 4859–4863.
27. Habib, G. M., Barrios, R., Shi, Z. Z., and Lieberman, M. W. (1996), *J. Biol. Chem.* **271,** 16,273–16,280.