ISSN 0582-9879 Acta Biochimica et Biophysica Sinica 2004, **36**(7): 485–491 **CN** 31-1300/Q

A Novel Gene Delivery System Targeting Urokinase Receptor

Xing-Hui SUN, Li TAN, Chun-Yang LI, Chang TONG, Jin FAN, Ping LI, and Yun-Song ZHU*

Department of Molecular Genetics, Key Laboratory of Molecular Medicine of Ministry Education, Shanghai Medical College of Fudan University, Shanghai 200032, China

Abstract Recombinant proteins that combine different functions required for cell targeting and intracellular delivery of DNA present an attractive approach for the development of nonviral gene delivery vectors. Here, we described a novel protein termed ATF-lys10 which facilitated cell-specific gene transfer via receptor-mediated endocytosis. ATF-lys10 was composed of the amino-terminal fragment of urokinase and ten lysines at the carboxyl terminus. Bacterially expressed ATF-lys10 protein existed in soluble form, and had antigenicity of human urokinase. Purified ATF-lys10 specifically bound to uPAR-expressing cells and formed protein-DNA complexes with plasmid pGL3-control. After neutralization of excess negative charge with poly-*L*-lysine, these complexes served as a specific gene delivery vector for uPAR-expressing cells. Lysosomotropic compounds, such as chloroquine, drastically increased the ATF-lys10 mediated gene delivery efficiency. Our results suggest that the recombinant protein ATF-lys10 with the properties of DNA binding and tumor cell targeting represents a promising method for gene transfer and expression in tumor cells.

Key words amino-terminal fragment; urokinase; gene transfer; endocytosis; urokinase receptor

Viral vectors are widely used in gene therapy due to their high efficiency of gene transfer. However, major disadvantages of viral vectors for gene transfer include the limitation of cell type specificity and the size of incorporated DNA, the potential risk of mutagenesis, induction of toxic or immunological reactions, and the reversion to replication competence. Nonviral delivery systems for gene transfer are being developed, including receptor-mediated gene transfer systems. Such vectors are usually prepared chemically by covalent linkage of functional components with properties of DNA binding and cell targeting. Cationic polymers, such as poly-*L*-lysine (PLL) [1] and polyethylenimine (PEI) [2], are able to bind plasmid DNA and protect it from enzymatic degradation, while folic acid [3], transferrin [4], mannose [5], galactose [6], epidermal growth factor [7] and oligopeptide ligand [8] are used for cell targeting moieties.

In this study, we produced a recombinant protein A TF-lys10 for gene delivery, which is composed of amino-terminal fragment (ATF) of urokinase (uPA) for binding uPA receptor (uPAR) and ten lysines at the carboxyl terminus of ATF for condensing plasmids.

Received: April 5, 2004 Accepted: May 7, 2004

This work was supported by a grant from the National Natural Science Foundation of China (No. 30170398)

*Corresponding author: Tel, 86-21-54237278; Fax, 86-21-64033738; E-mail, yszhu@shmu.edu.cn

Materials and Methods

Plasmids construction

Primers used in plasmids construction were listed in Table 1. The open reading frame of ATF was amplified from plasmid pZWE-ATF-PAI2CD [9], using primer 1 and primer 2. This 425-bp fragment has *Eco*RI and *Stu*I site at 5′ and 3′ respectively. The plasmid containing the *ATF-lys10* sequence was produced by ligating the 425-bp *Eco*RI-*Stu*I fragment with pET32-*HA-K* [10], forming pET32-*ATF-lys10*. Plasmid pET32-*ΔATF-lys10* which encoded mutant ATF-lys10 without amino acids 1–67 of ATF, was generated by digestion of pET32-*ATF-lys10* with *Nco*I and then ligation. The gene fragment encoding ATF was obtained from pET32-*ATF-lys10* by PCR using primer 3 and primer 4. After digestion with *Eco*RI and *Hin*dIII, the fragment was inserted into plasmid pET-32a(+) (Novagen), resulting in plasmid pET32-*ATF*, which encodes ATF without 10 lysines at the carboxyl terminus.

cDNA encoding the full-length uPAR was generated by

Table1 Primers used in plasmids construction

Primer	Sequence
Primer1	5′-GAGGAATTCATGAGCAACGAACTTCACCAA-3′
Primer2	5′-GAAGGCCTTTTTTGCCGTCTGCGCAGTC-3′
Primer3	5′-AGGGATCCAGCAACGAACTTCACCAAG-3′
Primer4	5′-GCCAAGCTTTCAGTCTGCGCAGTCATGCACCATGCAC-3′
Primer5	5′-CGGAATTCATGGGTCACCCGCCG-3′
Primer6	5′-GAAGATCTTTAGGTCCAGAGGAGAGTGCCTCC-3′

The restriction sites were underlined.

PCR, using primer 5 and primer 6. After digestion with *Eco*RI and *Bgl*II, the PCR product was inserted into vector pcDNA3.1/myc-his(–)A (Invitrogen). The recombinant plasmid was named pcDNA3.1-*uPAR*.

Production of recombinant proteins

Plasmid pET32-*ATF-lys10*, pET32-*ATF* and pET32-*ΔATF-lys10* were transformed into *E. coli* strain BL21 (DE3), respectively. A well isolated colony was inoculated into LB medium containing 100 μg/ml ampicillin, and cultured under vigorous shaking at 37 °C. Expression of hexahistidine-tagged ATF-lys10, ATF and ΔATF-lys10 were induced by adding IPTG (isopropyl-β-*D*-thiogalactopyranoside) to a final concentration of 1 mM, and cultured for additional 3 h. Bacterial cells were harvested by centrifugation at 6000 r/min for 15 min at 4 °C. Cells were resuspended in binding buffer (0.02 M Tris-HCl, 0.5 M NaCl, 5 mM imidazole) containing 1 mM protease inhibitor PMSF, followed by sonication at 800 W with 2 second-cycles of alternate sonication and resting. Cell extracts were clarified by centrifugation for 40 min at 10,000 r/min and 4 °C. Supernatant was filtered with a 0.45 μm filter and submitted to affinity chromatography on a histidine-binding resin column. Unbound proteins were removed with the binding buffer, and recombinant proteins were eluted with the imidazole buffer. 10 mM DTT was added to the elution fractions, and the solution was placed at 4 °C for 2 days. After that, 1 mM GSSG was added, and the mixture was placed for another 2 days at 4 °C, and then was dialyzed against 0.01 M phosphate buffer containing 0.01 M NaCl, and stored at –70 °C.

Western blot analysis

Purified recombinant proteins were separated under reducing conditions by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membrane. The membrane was probed with polyclonal anti-human uPA antibody, followed by goat anti-mouse IgG. The band was visualized by incubation with DAB and H_2O_2 in 1×PBS.

Gel retardation experiment [11]

Plasmid pGL3-control was incubated with 20 μg of ATF-lys10, ΔATF-lys10 and ATF, respectively, in buffer (pH 7.4) containing 20 mM HEPES, 150 mM NaCl at room temperature. After 30 min, the mixture was separated in 0.8% agarose gel.

Cell lines and transfection

Strain 95D and 95C cells with different metastatic potential [12], isolated from human large-cell lung carcinoma cell line PLA-801, were provided by the Cell Culture Laboratory of Department of Pathology, Chinese PLA General Hospital, and cultured in RPMI 1640 medium containing 10% bovine calf serum, 100 u/ml penicillin and 50 μg/ml streptomycin at 37 °C in a humidified CO_2 incubator with 5% CO_2. Plasmid pcDNA3.1-*uPAR* and pEGFP-C3 (Clontech) were transfected into 95C cells or 95D cells with Lipofectamine™ reagent (Invitrogen) according to the manufacturer's instructions. 48–72 h after transfection, cells were used for gene delivery or subjected to microscopical observations.

Binding of ATF-lys10 to uPAR

Binding of ATF-lys10 to uPAR was determined by fluorescence-activated cell sorter analysis using 95D cells as previously described [13,14]. 4×10^6 trypsinized cells were treated for 3 min at room temperature with 50 mM glycine-HCl buffer, pH 3.0, containing 100 mM NaCl. The mixture was neutralized by the addition of 0.5 volume of 0.5 M HEPES, 100 mM NaCl, pH 7.4, and then washed three times with buffer A (phosphate-buffered saline containing 1 mg/ml bovine serum albumin). Cells were collected by centrifugation, and resuspended in 200 μl of buffer B (binding buffer, serum-free RPMI 1640 medium containing 20 mM HEPES and 1 mg/ml bovine serum albumin, pH 7.4) in the presence of 20 nM ATF-lys10, ATF, ΔATF-lys10, pro-uPA or IgG, respectively. Unbound

proteins were discarded, and cells were washed three times with buffer A and then treated with 3 μl of polyclonal antibody against human uPA in 50 μl of buffer B for 40 min followed by goat anti-mouse IgG as a secondary antibody. Fluorescence of cells was analyzed with FACScan.

Gene delivery using ATF-lys10/plasmid/PLL complex

Transfection was performed as described [15]. Gene delivery complexes were prepared by incubation of 10 μg of plasmid pGL3-control or pEGFP-C3 with ATF-lys10 in a buffer containing 50 mM HEPES, 150 mM NaCl, pH 7.5, for 60 min at room temperature. To facilitate condensation of the protein-DNA complex, poly-*L*-lysine HBr (Sigma) with a molecular weight range of 15–30 kD was slowly added to the complex at various final concentrations, and the mixture (total volume of 100 μl) was incubated for an additional 60 min. For transfection, 5×10^5 cells were acid-treated and washed with serum-free medium. 1 ml of RPMI 1640 medium containing the ATF-lys10/plasmid/PLL complex, 20 mM HEPES, 150 mM NaCl and 1 mg/ml BSA with or without 100 μM chloroquine (Sigma) was added into each well. After 4 h incubation at 37 °C in humidified air with 5% CO_2, the medium was removed and 2 ml of complete culture medium was added, and the cells were further incubated for 48 h. Various amounts of pro-uPA were added to the mixture to investigate its effect on gene transfer efficiency.

Luciferase assay

Luciferase assay was performed as described in the Technical Bulletin No. 281 of luciferase assay system from Promega. The culture medium was removed, and the cells were washed three times with 1×PBS and lysed with 500 μl passive lysis buffer for 15 min at room temperature. The lysates were cleared by centrifugation, and the protein content of each sample was determined by the Bicinchoninic Acid (BCA) assay. The luminescence of each sample was recorded three times by using a luminometer in triplicate. The luciferase activity was represented as the number of relative light units in 1 mg protein (RLU/mg protein).

Results

Design and production of ATF-lys10, ATF and ΔATF-lys10

The recombinant expression plasmids [Fig. 1(A)] were transformed into *E. coli* strain BL21(DE3) respectively.

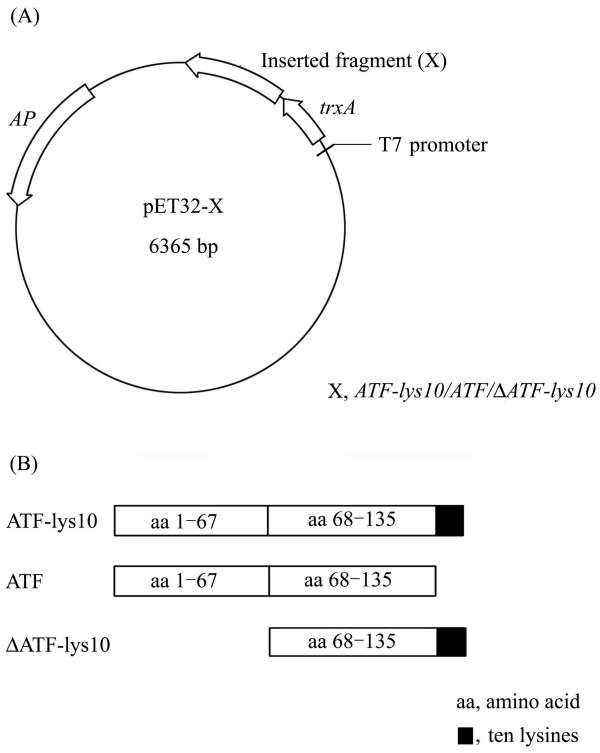

Fig. 1 (A) Structure of expression vectors pET32-*ATF-lys10*, pET32-*ATF* and pET32-*ΔATF-lys10*, and (B) schematic diagrams of recombinant proteins

(A) The recombinant gene fragment encoding ATF-lys10, ATF and ΔATF-lys10 were cloned into pET32-*HA-K* or pET-32a(+), and the obtained expression plasmids were named pET32-*ATF-lys10*, pET32-*ATF* and pET32-*ΔATF-lys10*. (B) ATF is the amino terminal fragment (aa 1–135) of uPA; In ATF-lys10, ten lysines were fused to the carboxyl terminus of ATF; ΔATF-lys10 is a mutant of ATF-lys10 lacking aa 1–67 of ATF. All of the three recombinant proteins were fused to the carboxyl terminus of thioredoxin (trx)-tag and His_6-tag.

After induction, there was a band of recombinant protein with the expected molecular weight, and most of the recombinant protein existed in soluble form. The recombinant proteins ATF-lys10, ATF and ΔATF-lys10, shown schematically in Fig. 1(B), were purified by one-step affinity chromatography purification. On the SDS-PAGE analysis, the purified proteins displayed one prominent band at the expected molecular mass respectively [Fig. 2 (A)]. Western blot analysis showed that the recombinant proteins had the antigenicity of human uPA [Fig. 2(B)].

Gel retardation experiment

Plasmid pGL3-control was incubated with ATF-lys10, ΔATF-lys10 and ATF at room temperature respectively. After 30 min, the sample was run in 0.8% agarose gel. ATF-lys10 and ΔATF-lys10 but not ATF could efficiently bind and retard plasmid pGL3-control (Fig. 3).

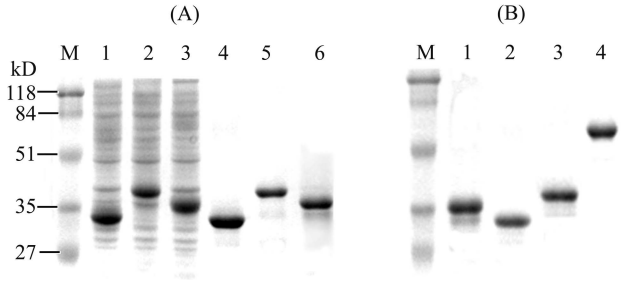

Fig. 2 SDS-PAGE and Western blot analysis of recombinant proteins

(A) SDS-PAGE analysis of recombinant proteins. M, protein marker; 1, 2 and 3, total protein of supernatant after sonication of *E. coli* strain BL21(DE3) respectively transformed with pET32-*ΔATF-lys10*, pET32-*ATF-lys10* and pET32-*ATF*; 4, 5 and 6, purified recombinant proteins ΔATF-lys10, ATF-lys10 and ATF respectively. (B) Western blot analysis of purified recombinant proteins; M, protein marker; 1, ATF; 2, ΔATF-lys10; 3, ATF-lys10; 4, human pro-uPA.

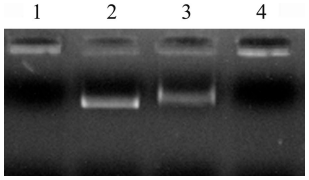

Fig. 3 Gel retardation experiment

0.2 μg plasmid pGL3-control was incubated with 20 μg of ATF-lys10, ΔATF-lys10 and ATF at room temperature respectively. After 30 min, the mixture was run in 0.8% agarose gel. 1, 0.2 μg plasmid pGL3-control + 20 μg ATF-lys10; 2, 0.2 μg plasmid pGL3-control; 3, 0.2 μg plasmid pGL3-control + 20 μg ATF; 4, 0.2 μg plasmid pGL3-control + 20 μg ΔATF-lys10.

uPAR-specific binding of ATF-lys10

It was reported that ATF could bind with high affinity to uPAR [16]. The function of ATF moiety in ATF-lys10 was investigated by fluorescence-activated cell sorter analysis to test if ATF-lys10 could also bind uPAR. 95D cells were incubated with ATF-lys10, ΔATF-lys10 and ATF respectively, and specifically bound proteins were detected with polyclonal antibody against human uPA followed by FITC-labeled goat anti-mouse IgG. The results were shown in Fig. 4. Efficient bindings of ATF-lys10 and ATF to uPAR were observed, but not ΔATF-lys10.

ATF-ly10 facilitates gene transfer into 95D cells

Plasmid pGL3-control was used to determine if ATF-lys10 could mediate gene transfer. The results were shown in Fig. 5(A,B). ATF-lys10 could mediate gene transfer, and the efficiency depended on the ratio of PLL/plasmid (*W*/*W*) and concentration of ATF-lys10. The transfection efficiency was very high when the ratio of PLL/plasmid was 12 [Fig. 5(A)], or at the final concentration of 5 nM ATF-lys10 [Fig. 5(B)]. With the increase of concentration of ATF-lys10, the transfection efficiency reduced [Fig. 5 (B)]. To investigate the transfection efficiency, 10 μg pEGFP-C3, 5 nM ATF-lys10 and 120 μg PLL were formed into complex as described in Methods. 95D cells cultured in six-well plates were treated with the gene delivery complex, and the results were shown in Fig. 5(C).

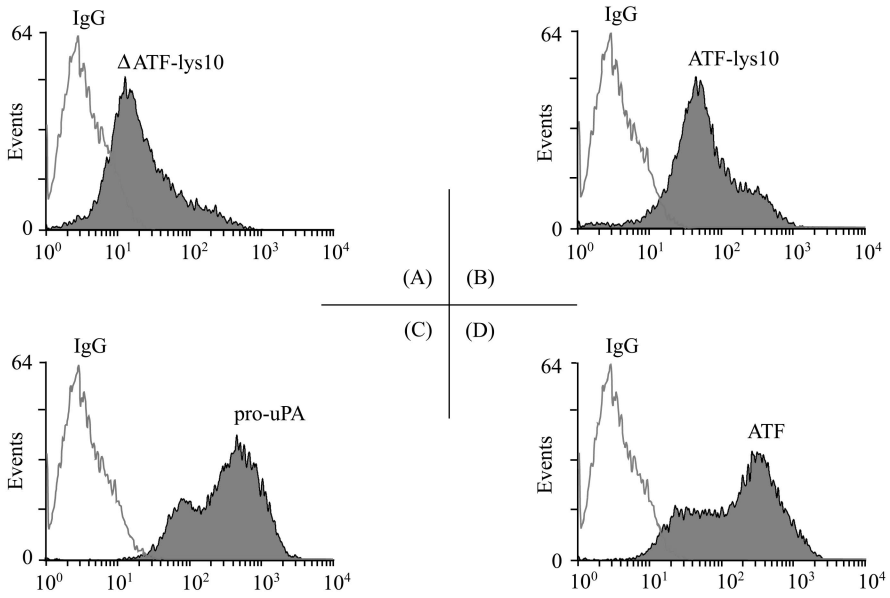

Fig. 4 Binding of recombinant proteins to uPAR

(A) 95D cell +ΔATF-lys10 + uPA polyclonal antibody + FITC-IgG. (B) 95D cell + ATF-lys10 + uPA polyclonal antibody + FITC-IgG. (C) 95D cell + pro-uPA + uPA polyclonal antibody + FITC-IgG. (D) 95D cell + ATF + uPA polyclonal antibody + FITC-IgG. They were all controlled with 95D cell + IgG +FITC-IgG.

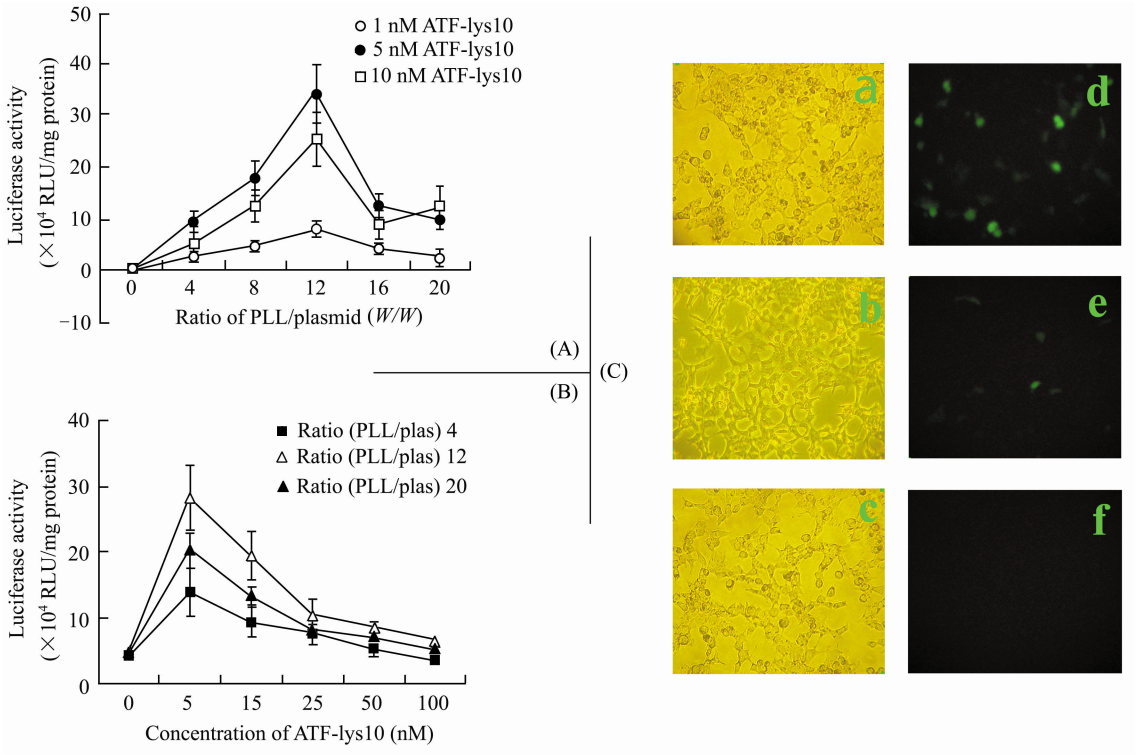

Fig. 5 ATF-lys10 mediated gene delivery in 95D cells

95D cells were seeded in 6-well tissue culture plates at a density of 5×10^5 cells/well and grown overnight. Transfection complexes containing indicated amounts of ATF-lys10, pGL3-control or pEGFP-C3 plasmid and PLL were prepared as described in "Materials and Methods" and added to the cells with 100 μM chloroquine. After incubation for 4 h, the medium was replaced with complete growth medium. After 48 h, cells were prepared for luciferase assay or visualized by fluorescence microscopy. (A) Gene delivery was affected by the ratio of plasmid/PLL (*W*/*W*); 1, 5 and 10 nM ATF-lys10, PLL and 10 μg plasmid pGL3-contral were assembled in complex with the indicated ratio of PLL/plasmid. (B) Gene delivery was affected by the concentration of ATF-lys10; with the indicated ratio of PLL/plasmid, PLL, plasmid and different amounts of ATF-lys10 were assembled in complex. (C) 95D cells were transfected with plasmid pEGFP-C3 using Lipofectamime™ Reagent (a, d) or ATF-lys10/pEGFP-C3/PLL complex (b, e), and visualized by fluorescence microscopy (d, e, f) or light microscope (a, b, c), panel c and f were 95D cells without transfection; data are representative of four experiments (200×). Each value is the mean ± SD of at least three independent experiments.

ATF-lys10 mediated gene delivery was uPAR-targeting

Recombinant protein ΔATF-lys10 and ATF were used to investigate if ATF-lys10 mediated gene delivery was uPAR targeting. The recombinant proteins were respectively assembled into gene delivery complex as described in "Materials and Methods". 95D cells were treated with the gene delivery complexes. We found that ATF-lys10 mediated gene delivery was more efficient than ATF and ΔATF-lys10 [Fig. 6(B)], which was inhibited by pro-uPA in dose-dependent manner [Fig. 6(A), column 1–5], but not low molecular weight uPA (lmw-uPA) which lacked the binding domain to uPAR [Fig. 6(A), column 6]. In the other hand, plasmid pGL3-control was transfected into 95D cells, 95C cells and 95C cells transfected with pcDNA3.1-*uPAR*. uPAR expression levels in the three group cells were detected by Western blot analysis [Fig. 6(D)]. The transfection efficiency was higher in 95D cells and 95C cells transfected with pcDNA3.1-*uPAR* than in 95C cells [Fig. 6(C)].

Discussion

Human uPA has 411 amino acids in length and is secreted as a 55 kD glycosylated protein. The binding domain is ATF (amino acid 1–135) which binds with high affinity (K_d=0.5 nM) to its receptor uPAR [16]. uPAR is overexpressed on a variety of tumors, including monocytic and myelogenous leukemia and cancers of the colon, breast, bladder, thyroid, liver, pleura, lung, pancreas, ovaries, head and neck [17]. Recently, it has been reported that ATF and ATF-toxin fusion protein can bind to uPAR with high affinity and be specifically internalized by uPAR-expressing cells [18]. So we produced a recombinant protein

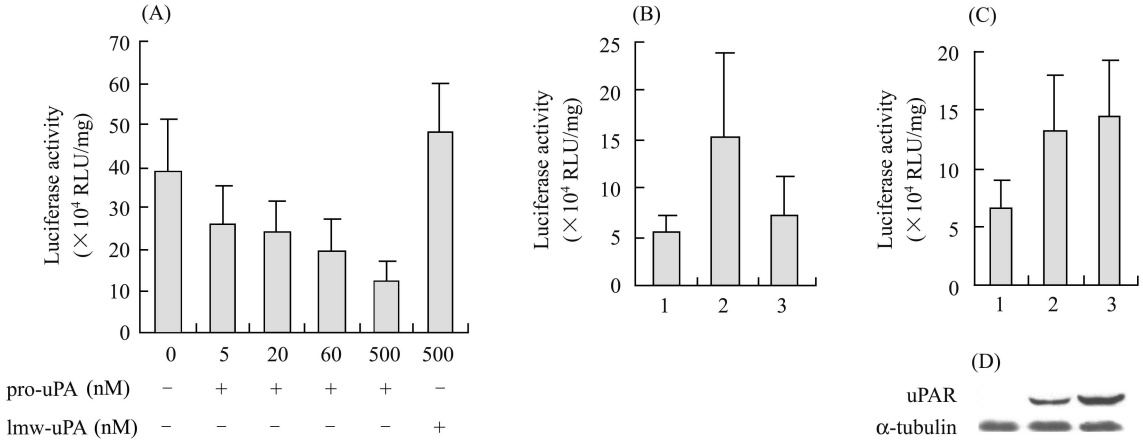

Fig. 6 ATF-lys10-mediated gene transfer is uPAR targeting

95D and 95C cells were seeded in 6-well tissue culture plates at a density of 5×10^5 cells/well and grown overnight. 95D cells were transfected with gene delivery complex containing ATF (B, column 1), ATF-lys10 (B, column 2) and ΔATF-lys10 (B, column 3) respectively. 95D cells in other wells were transfected with gene delivery complex ATF-lys10/pGL3-control/PLL, in the presence of pro-uPA (A, column 1–5) or lmw-uPA (A, column 6). Plasmid pcDNA3.1-*uPAR* was transfected into 95 cells with Lipofectamine™ Reagent. After 48 h, the cells (C, column 2) as well as 95C cells (C, column 1) and 95D cells (C, column 3) were transfected with gene delivery complex ATF-lys10/pGL3-control/PLL. 95C and 95D cells were used as control. Luciferase activity in all cells treated with gene delivery complex was measured after 48 h. uPAR protein levels in 95C cells, 95C cells transfected with pcDNA3.1-*uPAR* and 95D cells were shown in panel D. Each value is the mean ± SD of at least three independent experiments.

ATF-lys10 which was composed of ATF and ten lysines as a kind of nonviral vector for gene delivery. ATF-lys10 can bind to uPAR via ATF domain, and bind plasmid DNA via ten lysines.

ATF-lys10 was expressed in *E. coli* strain BL21(DE3) using the pET-32a(+) expression vector. This vector encodes a 109 aa thioredoxin (trx-tag) domain immediately upstream of His-tag sequence that allows easy purification. The biosynthesis of foreign protein is controlled by the T7lac promoter repressed by lacI, which is also carried in the vector. This sort of vector should be used only in bacterial strains containing a chromosomal copy of the gene for T7 RNA polymerase. In this study, induction by 1 mM IPTG resulted in the expression of recombinant proteins in soluble form. After one-step affinity chromatography purification, recombinant protein ATF-lys10 was obtained with high purity. The purified protein could bind plasmid DNA and bind uPAR. The ATF-lys10/PLL/plasmid complex could be internalized by 95D cells, resulting in luciferase gene expression, while ATF lacking ten lysines and ΔATF-lys10 lacking amino acids 1–67 of ATF could not mediate gene delivery efficiently [Fig. 6(B)]. The gene delivery efficiency in 95C cells was less than that in uPAR overexpression cells [Fig. 6(C)]. These data suggest that ATF-lys10 can mediate gene delivery, and ATF-lys10 mediated gene delivery is uPAR targeting.

Recombinant proteins ATF and ΔATF-lys10 were use as control to demonstrate that ATF domain and ten lysines in ATF-lys10 were indispensable for gene delivery. ATF could bind uPAR on cell surface [Fig. 4(D)], but could not mediate gene delivery efficiently because of lacking of ten lysines [Fig. 6(B)]. ΔATF-lys10 could bind plasmid (Fig. 3), but could not mediate gene delivery efficiently [Fig. 6(B)] because of lacking amino acids 1–67 in ATF which led to inability of ATF binding to uPAR [Fig. 4(A)]. These data indirectly suggest that ATF-lys10 mediated gene delivery is uPAR targeting.

Lysosomotropic compounds such as chloroquine increased the efficiency of receptor-mediated gene delivery. Chloroquine is a weak base known to raise the pH of acidic compartments of the endocytosis pathway. The unprotonated chloroquine can easily enter the lysosome. In the lysosome, the molecules become protonated as a result of the low pH which led to its accumulation. The accumulation caused the swelling of lysosomes and destabilization of their membranes [19]. This is why chloroquine can favor receptor mediated gene delivery. In this study, 100 μM chloroquine increased efficiency of gene delivery obviously. Hemagglutinin exists as a homotrimer of disulfide-bonded peptide chains HA1 and HA2. The highly conserved segment at the N-terminus of HA2 consists of approximately 20 amino acid residues. This hydrophobic segment is essential for membrane fusion and called “fusion peptide”. It has been demonstrated [4,20] that the fusion peptide can augment efficiency of receptor mediated gene transfer. In this study, recombinant protein

HA20-lys10 [10] did not increase gene transfer efficiency (data not shown), probably due to thioredoxin fused at its N-terminus which influence the structure change of "fusion peptide" under acid condition in lysosome.

In the gene delivery system, PLL was added to package plasmid DNA and neutralize its excess negative charge. The existence of PLL led to a nonspecific binding of plasmid/ATF-lys10 complex to cell, which could be seen in [Fig. 5(B), column 1]. In the presence of ATF-lys10, the gene transfer efficiency was improved obviously, but the expression of reporter gene could not be completely inhibited by pro-uPA [Fig. 6(A), column 5], this was probably in part because of the existence of PLL.

In summary, ATF-lys10 can mediate gene delivery but the efficiency is low. Our results demonstrated that ATF-lys10 with properties of DNA binding and cell targeting had the potential to be used as nonviral vector in gene transfer and expression.

Acknowledgements

We thank Dr. Xia WANG for kindly providing plamid pZWE-ATF-PAI2CD and polyclonal antibody against human uPA. We also thank professor Antonio Villaverde (Universitat Autonoma de Barcelona) for providing plasmid pGL3-control.

References

1 Perales JC, Ferkol T, Molas M, Hanson RW. An evaluation of receptor-mediated gene transfer using synthetic DNA-ligand complexes. Eur J Biochem, 1994, 226(2): 255–266

2 Lemkine GF, Demeneix BA. Polyethylenimines for *in vivo* gene delivery. Curr Opin Mol Ther, 2001, 3(2): 178–182

3 Sudimack J, Lee RJ. Targeted drug delivery via the folate receptor. Adv Drug Deliv Rev, 2000, 41(2): 147–162

4 Wagner E, Plank C, Zatloukal K, Cotten M, Birnstiel ML. Influenza virus hemagglutinin HA-2 N-terminal fusogenic peptides augment gene transfer by transferrin-polylysine-DNA complexes: Toward a synthetic virus-like gene-transfer vehicle. Proc Natl Acad Sci USA, 1992, 89(17): 7934–7938

5 Diebold SS, Plank C, Cotten M, Wagner E, Zenke M. Mannose receptor-mediated gene delivery into antigen presenting dendritic cells. Somat Cell Mol Genet, 2002, 27(1-6): 65–74

6 Perales JC, Grossmann GA, Molas M, Liu G, Ferkol T, Harpst J, Oda H *et al*. Biochemical and functional characterization of DNA complexes capable of targeting genes to hepatocytes via the asialoglycoprotein receptor. J Biol Chem, 1997, 272(11): 7398–7407

7 Kircheis R, Blessing T, Brunner S, Wightman L, Wagner E. Tumor targeting with surface-shielded ligand-polycation DNA complexes. J Control Release, 2001, 72(1-3): 165–170

8 Lee TK, Han JS, Fan ST, Liang ZD, Tian PK, Gu JR, Ng IO. Gene delivery using a receptor-mediated gene transfer system targeted to hepatocellular carcinoma cells. Int J Cancer, 2001, 93(3): 393–400

9 Wang X, Li P, Zhang YQ, Hou M, Sun XH, Tan L, Zhu YS. Biological function of fusion protein ATF-PAI2CD. Acta Biochim Biophys Sin, 2003, 35(7): 624–628

10 Sun XH, Zhang YQ, Wang X, Hou M, Tan L, Li P, Zhu YS. Fusion expression, purification of HA2-Lys10 in *E. coli* and its gel retardation experiments. Fudan Univ J Med Sci, 2004, 31: 32–35

11 Dai FH, Chen Y, Ren CC, Li JJ, Yao M, Han JS, Gong Y *et al*. Construction of an EGF receptor-mediated histone H1(0)-based gene delivery system. J Cancer Res Clin Oncol, 2003, 129: 456–462

12 Lu YL, Wang JX, Li XH, Li HF, Chen LZ, Li WH. Spontaneous metastasis of clonal cell subpopulations of human lung large cell carcinoma after subcutaneous inoculation in nude mice. Chin J Oncol, 1989, 11: 3–7

13 Stoppelli MP, Tacchetti C, Cubellis MV, Corti A, Hearing VJ, Cassani G, Appella E *et al*. Autocrine saturation of pro-urokinase receptors on human A431 cells. Cell, 1986, 45(5): 675–684

14 Uherek C, Fominaya J, Wels W. A modular DNA carrier protein based on the structure of diphtheria toxin mediates target cell-specific gene delivery. J Biol Chem, 1998, 273(15): 8835–8841

15 Wagner E, Cotten M, Foisner R, Birnstiel ML. Transferrin-polycation-DNA complexes: The effect of polycations on the structure of the complex and DNA delivery to cells. Proc Natl Acad Sci USA, 1991, 88(10): 4255–4259

16 Cubellis MV, Nolli ML, Cassani G, Blasi F. Binding of single-chain prourokinase to the urokinase receptor of human U937 cells. J Biol Chem, 1986, 261(34): 15819–15822

17 Liu S, Bugge TH, Leppla SH. Targeting of tumor cells by cell surface urokinase plasminogen activator-dependent anthrax toxin. J Biol Chem, 2001, 276(21): 17976–17984

18 Rajagopal V, Kreitman RJ. Recombinant toxins that bind to the urokinase receptor are cytotoxic without requiring binding to the alpha(2)-macro-globulin receptor. J Biol Chem, 2000, 275(11): 7566–7573

19 Wattiaux R, Laurent N, Wattiaux-De Coninck S, Jadot M. Endosomes, lysosomes: Their implication in gene transfer. Adv Drug Deliv Rev, 2000, 41(2): 201–208

20 Schoen P, Chonn A, Cullis PR, Wilschut J, Scherrer P. Gene transfer mediated by fusion protein hemagglutinin reconstituted in cationic lipid vesicles. Gene Ther, 1999, 6(5): 823–832

Edited by
Ding-Gan LIU