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ORIGINAL ARTICLE

A novel method for promoting heterologous protein expression in *Escherichia coli* by fusion with the HIV-1 TAT core domain

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Abstract The human immunodeficiency virus type 1 (HIV-1) transactivator of transcription (TAT) protein, a member of the protein transduction domain (PTD) superfamily, can deliver heterologous proteins across most biomembranes without losing bioactivity. However, there is no report on whether the TAT core domain containing the sequence 'YGRKKRRQRRR' has other functions. As the TAT core domain is most basic (pI = 12.8) and has biomembrane crossing ability, we hypothesized it might probably influence the protein expression level due to subcellular redistribution of target proteins in the cells. To address this issue, we constructed the prokaryotic expression vector pET28b-TAT-EGFP (using the vector pET28b-EGFP for control) containing the core domain coding region, and transformed the vector into *E. coli* BL21 (DE3) cells for expression of the enhanced green fluorescent protein (EGFP) with the inducer isopropyl-β-D-thiogalactopyranoside (IPTG). Equal amount of the total proteins were fractionated using 15% SDS-PAGE and identified by western blot, and the plasmid copy number was assayed by Southern blot. In order to further study the subcellular localization of heterologous proteins in *E. coli* cells, the cytoplasmic and periplasmic components were extracted by chloroform and osmotic shock techniques. Interestingly, our data showed that the TAT core domain was not only able to promote the heterologous protein expression in *E. coli*, but also improve the yields and the solubility of heterologous proteins, while the plasmid copy number of TAT-containing clones and TAT-free clones was not affected by the TAT core domain. In addition, the TAT-tagged protein was mainly localized in the cytoplasm and also accumulated in the periplasmic space along with the time for protein expression, while in contrast, the TAT-free protein was mainly expressed in the periplasm and only a few in cytoplasm. A further examination on the distribution of the expressed proteins in cytoplasm and periplasm suggested that the TAT core domain might promote protein expression in the cytoplasm initially and then partially deliver them across the cytomembrane to the periplasmic space in a concentration-dependent manner. Taken together, our current data have provided a novel method for improving heterologous protein expression in prokaryotic cells by fusion with the TAT core domain, which will promote expression efficiency of bioactive proteins for protein engineering.

Keywords HIV-1 TAT domain · Prokaryotic expression · Plasmid copy number · Cytoplasm · Periplasm · *E. coli*

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Introduction

In the past several decades, a variety of eukaryotic and prokaryotic expression systems have been introduced for the expression of heterologous proteins. Prokaryotic expression systems are still widely used because they are easy to prepare and transform, grow quickly, produce adequate amounts of proteins, and only need inexpensive equipments for growth and storage. However, the deficiencies of *E. coli* expression systems are occasional low yields and difficulty to obtain soluble proteins because large amounts of expressed proteins tend to yield inactive and insoluble aggregates due to failure of protein folding process (Schein 1991). Therefore, a number of approaches have been

introduced to promote the yield and solubility of heterologous proteins, including induction of protein expression at lower temperatures (15–30°C), induction with lower concentrations of inducer as well as induction for longer time, cultivation in minimal media, and transportation to periplasmic space, etc. (Neu and Heppel 1964; Nossal and Heppel 1966; Hopper et al. 1985; Marvin and Witholt 1987; Blackwell and Horgan 1991; Burton et al. 1991; Dubendorff and Studier 1991; Vazquez-Laslop et al. 2001). However, despite these numerous approaches, it is still necessary to find more strategies to promote protein expression because no single technique works well for all proteins. Recently, we found that the hendeca polypeptide encoded by human immunodeficiency virus type 1 (HIV-1), transactivator of transcription (TAT) core domain, markedly increased the yield of heterologous proteins and also increased their solubility, suggesting an alternative choice for promoting heterologous protein expression in prokaryotic cells.

The TAT protein is a member of the protein transduction domain (PTD) superfamily, which includes HIV-1 TAT peptide, homeodomain of *Drosophila Antennapedia* (Antp), HSV VP22 transduction factor, and synthetic polylysine/polyarginine (Joliot et al. 1991; Derossi et al. 1994; Prochiantz 2000; Jin et al. 2001). It has a highly basic amino acid core domain (YGRKKRRQRRR) and can traverse most biomembranes either alone or fused with other proteins or peptides. It has been reported that the transduction of TAT peptide conjugation proteins is not a classical receptor/transporter-dependent process or an endosome-mediated manner, but may be a concentration-dependent or temperature- and time-dependent pattern (Frankel and Pabo 1988; Kwon et al. 2000; Lindsay 2002). A large number of studies have shown that the TAT peptide can deliver many macromolecules across membrane, such as proteins, nucleic acids, nanoparticles, and liposomes across biomembranes (Fawell et al. 1994; Nagahara et al. 1998; Schwarze et al. 1999; Watson and Edwards 1999; Kwon et al. 2000; Lewin et al. 2000; Schwarze and Dowdy 2000; Caron et al. 2001; Jin et al. 2001; Torchilin et al. 2001; Wills et al. 2001; Torchilin 2002; Ryu et al. 2003; Ryu et al. 2004; Snyder and Dowdy 2004; Wu et al. 2006). However, there is no report on other novel functions of the TAT peptide. In our pilot study for exploring the transduction function, we unexpectedly found that the TAT core domain could probably promote the expression of heterologous proteins in *E. coli*. Most surprisingly, we found that the TAT core domain was not only able to accelerate expression of heterologous proteins in *E. coli*, but also promoted the yield and the solubility of heterologous proteins, although the plasmid copy number was not significantly affected. The illustration of this topic will probably provide a novel method for improving heterologous protein expression in prokaryotic cells.

Materials and methods

Construction of prokaryotic expression vectors

The prokaryotic expression vectors pET28b-TAT-EGFP and pET28b-TAT were kindly provided by Dr. Xiaoming Yang in our institute. The EGFP cDNA fragment was amplified from the plasmid pET28b-TAT-EGFP by PCR using the primers, 5′-CATG*CCATGG*ATGGTGAGCAAG GGCGA-3′ (underlined for *Bam*HI site) and 5′-CCG*CTC GAG*CTTGTACAGCTCGTCC-3′ (underlined for *Xho*I site), and subcloned into the prokaryotic expression vector pET28b-TAT and pET28b. All the plasmid constructs were confirmed by direct sequencing.

Expression of the proteins

The plasmids of pET28b-TAT-EGFP and pET28b-EGFP were transformed into *E. coli* BL21 (DE3) cells, respectively, and incubated in a 37°C incubator for 10–12 h for positive clones growing. After randomly picking up one clone in each vector constructs (TAT-vector or TAT-free vector) and inoculating in 5 ml LB medium containing kanamycin (100 μg/ml), following with shaking at 37°C, 220 rpm to logarithmic growth phase, the bacterial cells was diluted to $OD_{600} = 0.8$. Five milliliters of bacterial cells (about 3.2×10^8 cells) was inoculated in 150 ml LB medium containing kanamycin (100 μg/ml) and incubated at 37°C, 220 rpm for 4–5 h until $OD_{600} = 0.6$–1.0. The incubation was followed by IPTG (1 mM) induction at 30°C for 6 h. The bacterial cells with induction for protein expression for 0, 2, 4 and 6 h were harvested and subjected to sonication in ice-cold PBS, and then centrifuged at 12,000 rpm for 10 min and filtered by passage through a 0.45 μm filter. The equal volume samples were then prepared and fractionated by 15% SDS-PAGE and western blot. In order to further demonstrate the TAT-induced protein expression in more clones, we further randomly picked up eight clones in the pET28b-TAT-EGFP1 group as well as the pET28b-EGFP2 group and examined the protein expression as mentioned above. All experiments were repeated at least three times.

Assay of the plasmid copy number by Southern blot

In order to determine whether the difference of protein expression between TAT-tagged protein and TAT-free protein are caused by different amounts of plasmids in the analyzed clones, we assayed the plasmid copy number by Southern blot. At first, we designed the oligonucleotide probe, 5′-ATGGCTAGCATGACTGGTGGACAGCAAAT GGGT-3′, and labeled the 5′-terminal first oligonucleotide with biotin. Subsequently, the plasmid vector pET28b-TAT-EGFP and pET28b-EGFP were transformed into the

E. coli BL21 (DE3) cells respectively and incubated in a 37°C incubator for 10–12 h until positive clones were growing. After picking one clone in each group and inoculating in 5 ml LB medium containing kanamycin (100 μg/ml), the *E. coli* cells were allowed to grow with shaking at 37°C, 220 rpm to logarithmic growth phase. Later, the bacterial cells were diluted to $OD_{600} = 0.8$ and 4 ml bacteria cells (about 2.5×10^8 cells) was used for extraction of the plasmid DNA. Equal amount of plasmid DNA was cut by *Apa*I restriction endonuclease and fractionated by 0.7% agarose gel. The plasmid DNA was denatured into single strands and transferred to nitrocellulose (NC) membrane for prehybridization at 65°C for 4–6 h followed by hybridization with the abovementioned oligonucleotide probe at 65°C for 8–16 h. Finally, the NC membrane was washed with washing buffer and incubated with HRP-streptavidin (3 μg/ml) at room temperature for 1 h and washed for three times (10 min for each time). Chemiluminescence substrate luminal reagent (GE Healthcare) and exposure to X-ray film were used to examine the immunolabeled bands. The optical density of the bands was then quantified with the ImageJ program as mentioned above.

Extraction of periplasmic proteins and cytoplasmic proteins

The bacterial cells with induction for protein expression for 0, 2, 4 and 6 h were harvested. The periplasmic proteins were extracted by chloroform and osmotic shock techniques as described by Ames et al. (1984). The cytoplasmic proteins were also extracted by sonication. The equal volume samples were prepared and fractionated by 15% SDS-PAGE and determined by western blot. All experiments were repeated at least three times.

Western blot

Equal volume samples were fractionated by electrophoresis through 15% polyacrylamide gels and transferred to a polyvinylidene difluoride (PVDF) membrane (GE Healthcare) following the manufacturers' instructions. The membrane was probed with the first antibody, mouse-derived anti-GAPDH antibody (1:500 in TBST, Beijing Zhongshan Biotechnology) or mouse-derived anti-His antibody (1:3,000 in TBST, Sigma), for 1.5 h at room temperature, followed by incubation of the HRP-conjugated goat anti-mouse secondary antibody (1:5,000 in TBST, Beijing Zhongshan Biotechnology), and incubated at room temperature for 1 h. Chemiluminescence substrate luminal reagent (GE Healthcare) and exposure to X-ray film were used to examine the immunolabeled bands. The optical density of the bands was scanned and quantified with the ImageJ program as mentioned above.

Statistical analysis

The statistical analysis was done with the SPSS software (version 13.0, SPSS, USA) using Student's *t* test with significant differences defined at $P < 0.05$.

Results

Preparation of the prokaryotic expression vectors

In our pilot study by using the TAT core domain which only contains 11 amino acids, 'YGRKKRRQRRR', we unexpectedly found that this TAT tag was probably able to promote the expression of heterologous proteins. To further

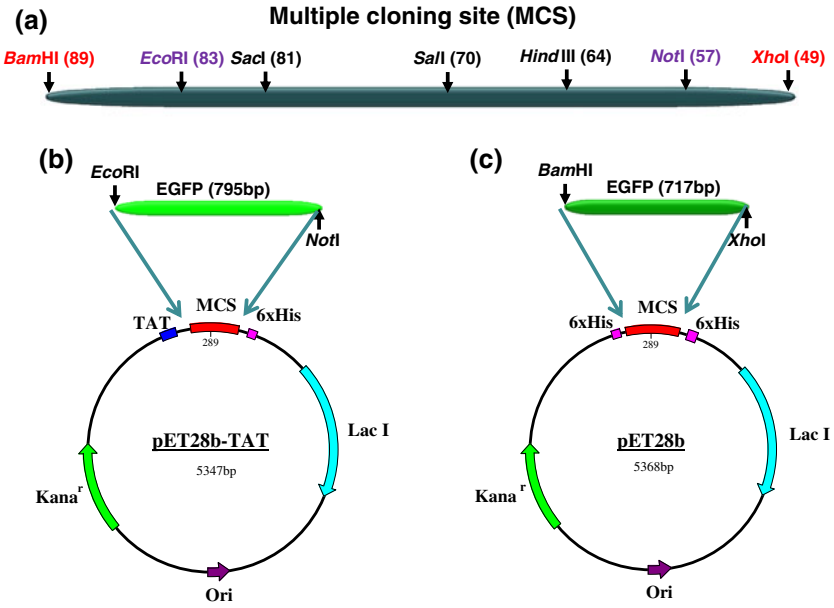


Fig. 1 Schematic diagram showing preparation of the prokaryotic expression vectors. **a** Multiple cloning site (MCS) of the vectors; **b** construction of the vector pET28b-TAT-EGFP; **c** construction of the vector pET28b-EGFP

characterize the potential use of the TAT core domain as a fusion tag, we constructed the prokaryotic expression vector pET28b-TAT-EGFP (using the vector pET28b-EGFP for control) with the coding sequence of this tag in frame (Fig. 1; Table 1), and confirmed by direct sequencing.

The TAT core domain can promote heterologous protein expression in *E. coli*

When the EGFP proteins with or without the TAT tag were induced with the inducer isopropyl-β-D-thiogalactopyranoside (IPTG), we could directly observe the visible fluorescent signal even under the sun light with human naked eyes (data not shown). To further observe the fluorescent signal diversity between TAT-EGFP and EGFP alone, the green fluorescent images were obtained by fluorescent microscope, differential interference contrast (DIC) microscopy and then merged. Two empty vectors, pET28b-TAT and pET28b, were used as control. Our data showed that the fluorescent signal of TAT-EGFP was stronger than that of EGFP (without TAT tag), and both the controls did not have any fluorescent signal (Fig. 2a). In addition, the fluorescent signal of TAT-EGFP was remarkably stronger than that of EGFP, consistent with that of the single cell signals (Fig. 2b).

To further investigate the TAT tag functions for promoting heterologous protein expression in *E. coli*, the bacterial cells were harvested at different time including 0 h (control), 2, 4 and 6 h of induction followed with preparation of total proteins, the supernatant proteins and the sediment proteins. Equal amount of protein samples were fractionated by 15% SDS-PAGE and western blot assay with the bacteria house-keeping protein glyceraldehyde phosphate dehydrogenase (GAPDH) as an internal standard (Fig. 3a, b). The images were analyzed using ImageJ program (http://rsb.info.nih.gov/ij/, NIH) and further plotted by Origin 8.0 software. We found that the integrated optical density (IOD) of TAT-EGFP was significantly higher than that of EGFP ($P < 0.01$), and both the two proteins, with or without TAT tag, were mainly localized in supernatant and only a minor amount in

Table 1 Construction of the prokaryotic expression vectors used in this study

Prokaryotic expression vectors	cDNA fragment coding for heterologous protein	Size of the coding region for the fusion protein (bp)	Fusion protein to be expressed	Size of the fusion protein to be expressed (kDa)
pET28b-TAT-EGFP1	*Eco*RI-EGFP1-*Not*I	795	TAT-EGFP1-6 × His	33.2
pET28b-EGFP2	*Bam*HI-EGFP2-*Xho*I	717	6 × His-EGFP2-6 × His	30.7

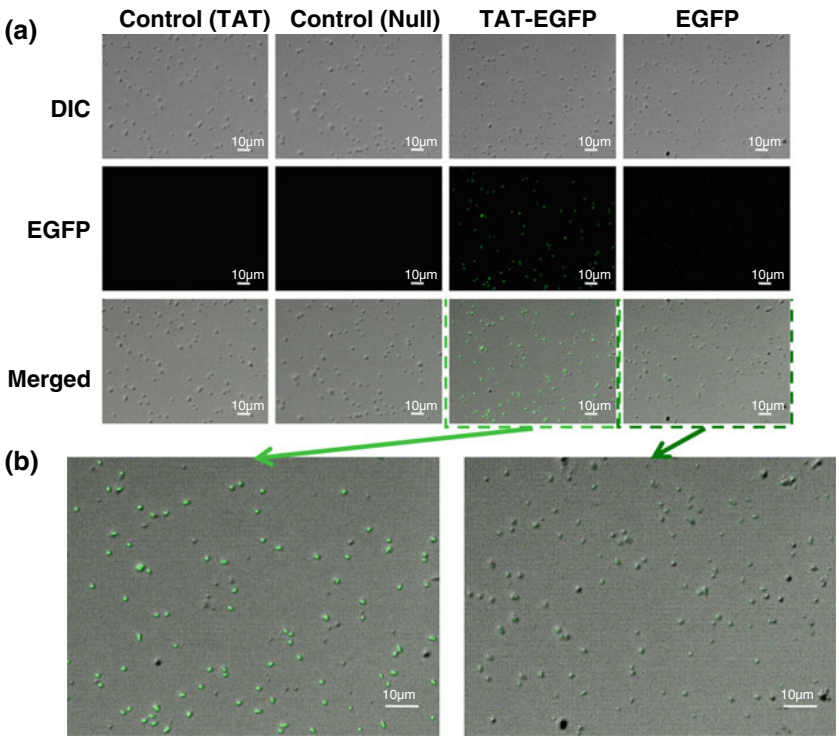


Fig. 2 Analysis of fluorescent signals of the TAT-EGFP and EGFP proteins by fluorescent microscopy. **a** Acquisition and merging of the differential interference contrast (DIC) microscopy images and the fluorescent images; **b** amplification for 2 × fold of the merged images of TAT-EGFP and EGFP respectively. 'Control (Null)' indicated the empty vector pET28b. 'Control (TAT)' indicated the empty vector pET28b-TAT

sediment. Moreover, the expression of TAT-EGFP was rapidly increasing and even peaked to a stable phase when induced for 4 h, but at that time the EGFP protein was only slowly and gradually increasing. Despite the yields of TAT-tagged protein, TAT-EGFP, was significantly increased, its solubility was also unaffected. Clearly, our data here demonstrated that the TAT core domain was not only able to markedly promote heterologous protein expression, but also improve the solubility of heterologous proteins (Fig. 3c).

To further determine whether this is an occasional phenomenon or not, the plasmids pET28b-TAT-EGFP and pET28b-EGFP were transformed into *E. coli* cells respectively followed with randomly picking up eight individual clones of TAT-tagged and TAT-free, and then randomly paired to eight groups (a TAT-clone vs. a TAT-free clone as a paired group). The protein expression levels at induction of 0, 2, 4 and 6 h were then analyzed by 15% SDS-PAGE (Fig. 4a) as mentioned above. Our data clearly showed that the protein expression level of all of the TAT-tagged proteins were significantly higher than that of the paired TAT-free proteins (Fig. 4b, $P < 0.01$), suggesting that the TAT core domain can markedly promote heterologous proteins expression.

Promotion of heterologous protein expression in *E. coli* by the TAT core domain was not due to different amounts of transformed plasmid DNA

Although it is clear that the TAT core domain can significantly promote heterologous protein expression, we did not know whether the promotion was due to different amounts of transformed plasmid DNA in *E. coli* cells. To address this issue, we further compared the plasmid copy number between the *E. coli* cells containing the TAT-vectors and TAT-free constructs by Southern blot followed with image analysis using the ImageJ program as mentioned above. Our data showed that there was no significant difference of the plasmid copy number between the *E. coli* cells containing the pET28b-TAT-EGFP1 vectors and pET28b-EGFP2 constructs (Fig. 5). Therefore, it seemed that promotion of heterologous protein expression in *E. coli*

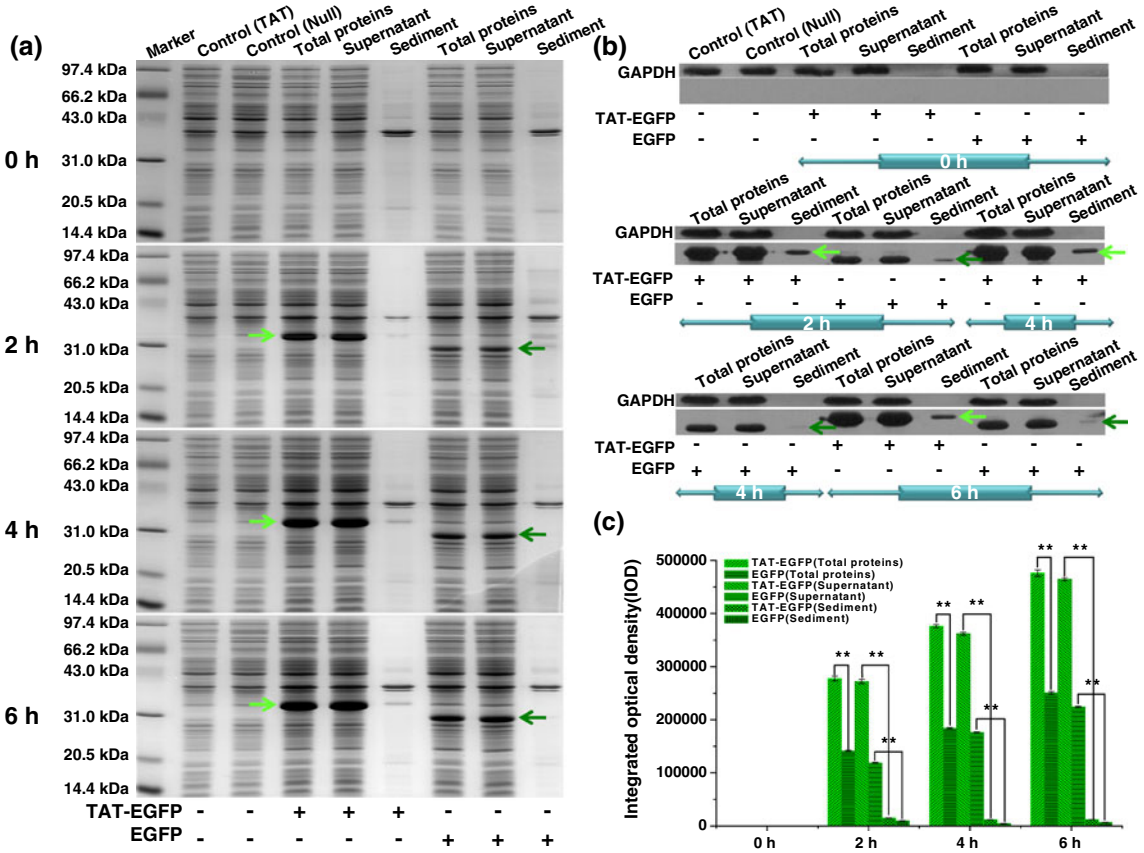
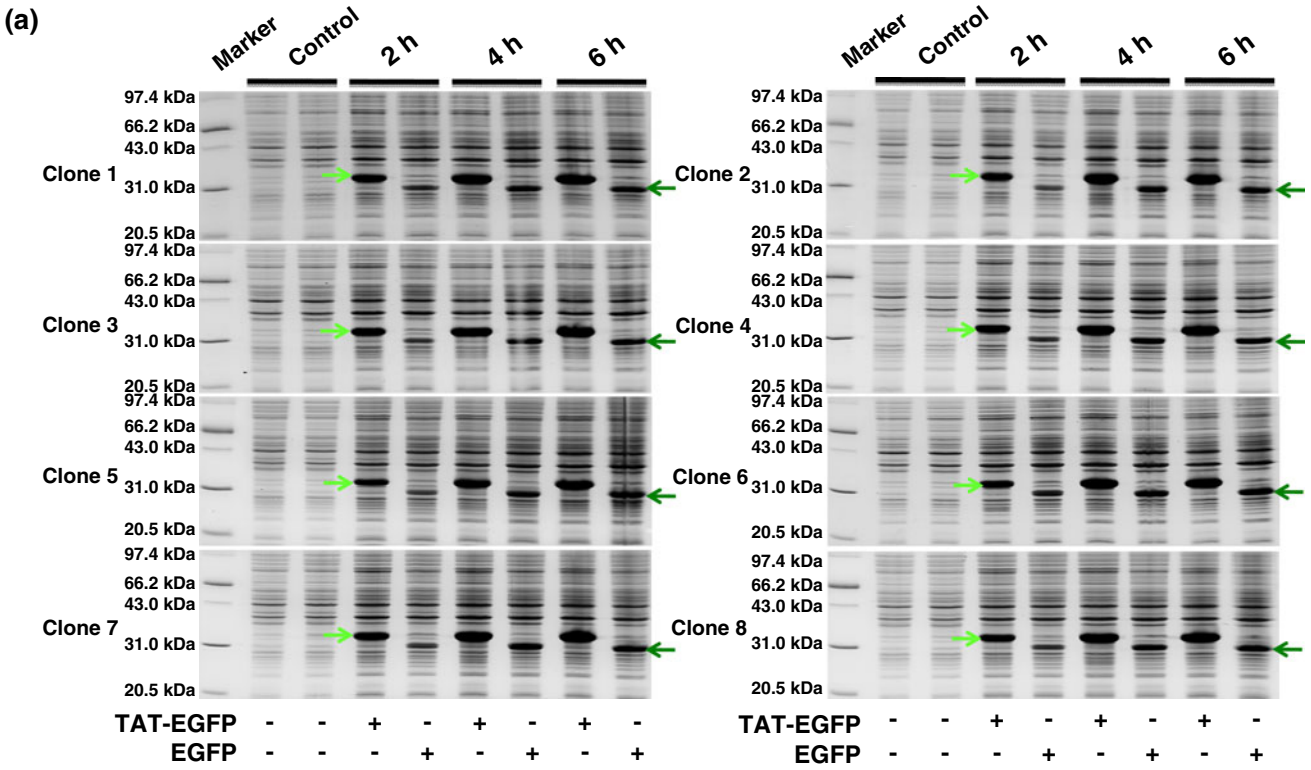
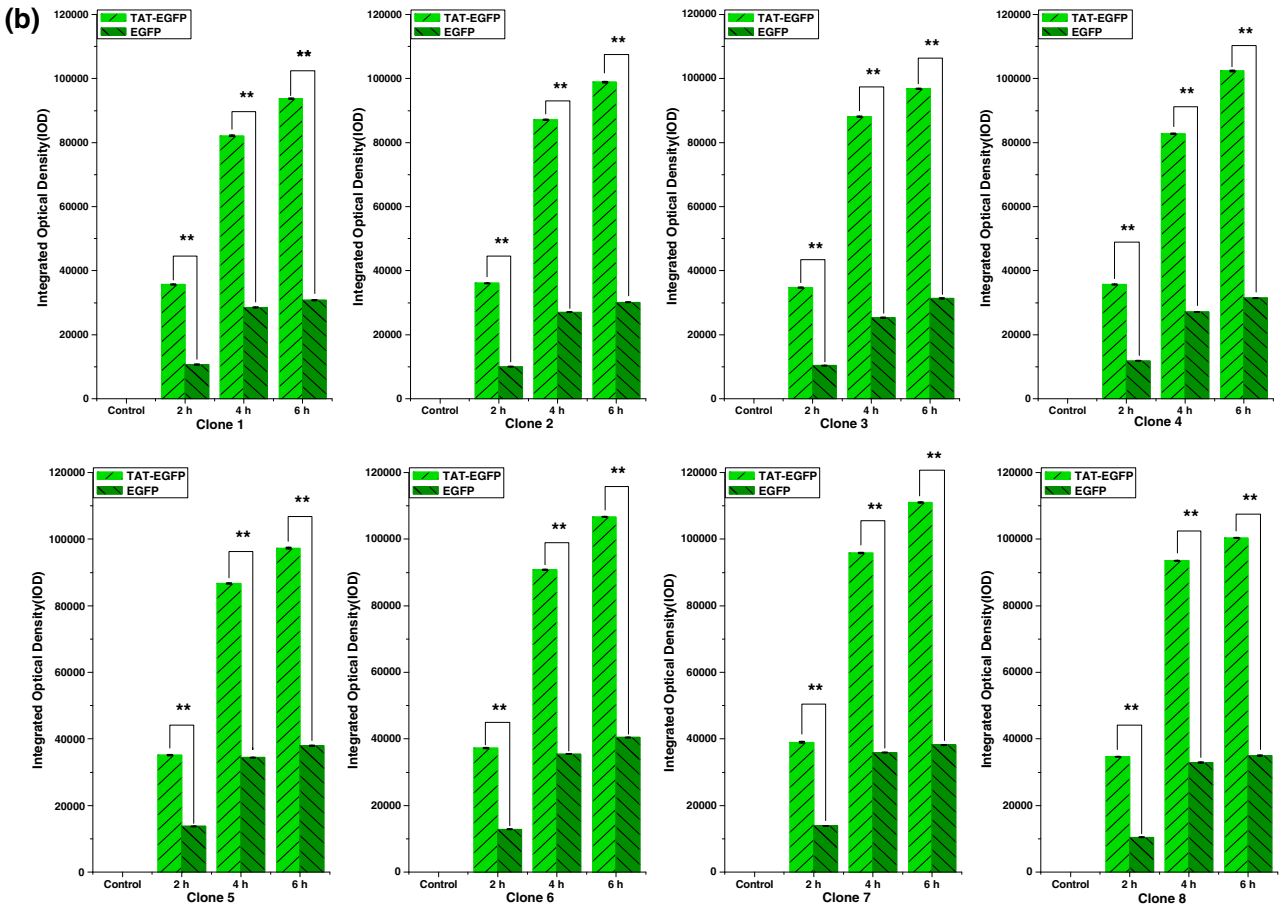


Fig. 3 Comparison of the expression levels between the TAT-EGFP and TAT-free proteins. **a**, **b** SDS-PAGE and western blot analysis of the total proteins, the supernatant proteins and the sediment proteins using GAPDH as a standard for equal total cells at different expression time; **c** histogram showing the diversity between TAT-EGFP and EGFP at different expression time. 'Control (Null)' indicated the empty vector of pET28b. 'Control (TAT)' indicated the empty vector of pET28b-TAT. Arrowheads indicated TAT-EGFP (higher band) or EGFP (lower band). $^{**}P < 0.01$, compared to EGFP


(a)
Marker
Control
2 h
4 h
6 h
97.4 kDa
66.2 kDa
43.0 kDa
31.0 kDa
20.5 kDa
Clone 1
Clone 2
Clone 3
Clone 4
Clone 5
Clone 6
Clone 7
Clone 8
TAT-EGFP
EGFP


(b)
TAT-EGFP
EGFP
Integrated Optical Density(IOD)
Control
2 h
4 h
6 h
Clone 1
Clone 2
Clone 3
Clone 4
Clone 5
Clone 6
Clone 7
Clone 8

◀ **Fig. 4** Protein expression level assay of further randomly picked eight clones. **a** SDS-PAGE analysis of expression level of total proteins of equal total cells at different expression time. **b** Histogram showing the diversity between TAT-EGFP and EGFP at different expression time. 'Control' indicated the 0 h induction. Arrowheads indicated TAT-EGFP (higher band) or EGFP (lower band). $^{**}P < 0.01$, compared to EGFP

by the TAT core domain was not due to different amounts of transformed plasmid DNA, but rather due to an unknown mechanism to be further investigated.

Subcellular localization of the TAT-tagged proteins and TAT-free proteins in *E. coli*

The *E. coli* cells contained two typical subcellular structures including cytoplasm and periplasm, which were the essential places for heterologous protein expression (Baecker et al. 1978; Beacham 1979; Ames et al. 1984). Although the TAT core domain had significant biomembrane crossability, however, our examination on the culture medium did not reveal any TAT-tagged proteins (data not shown). To explore the subcellular localization of TAT-tagged proteins and TAT-free proteins, the bacterial cells with the induction on protein expression for 0, 2, 4 and 6 h were harvested and extracted with chloroform and osmotic shock techniques. The periplasmic and cytoplasmic components were then fractionated by 15% SDS-PAGE followed by western blot analysis respectively. Our result revealed that the TAT-tagged protein was mainly expressed in the cytoplasm and also increased in the periplasmic space along with the time of expression, while the TAT-free protein was mainly localized in periplasm and only a minor amount in cytoplasm (Fig. 6).

Taken together we hypothesized that the TAT core domain might markedly promote heterologous protein expression in cytoplasm initially and then partially delivering the expressed proteins across the cytomembrane to the periplasmic space along with the time of expression in a concentration-dependent manner (Fig. 7, left panel). However, the TAT-free protein, the EGFP alone, was also able to be transported to the periplasmic space with an unknown mechanism, although the special protein transportation system was probably involved but need further investigation (Fig. 7, right panel).

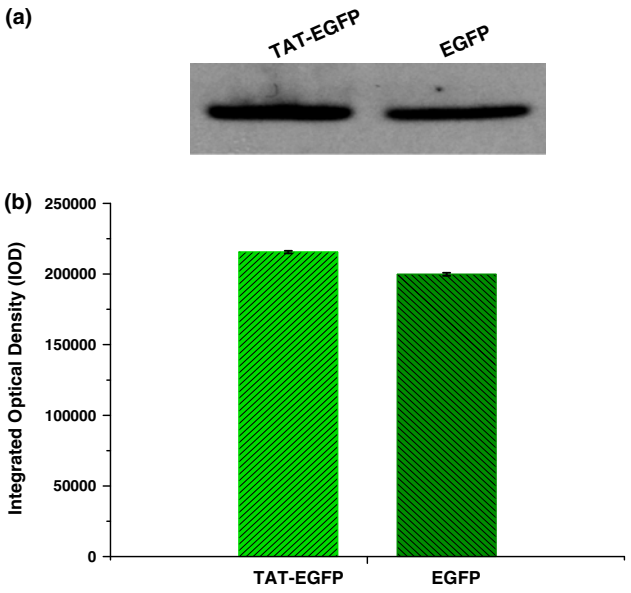


Fig. 5 Southern blot assay of the plasmid copy number between the *E. coli* cells transformed with the TAT-EGFP vectors and TAT-free constructs. **a** Southern blot showed that there was no significant difference of the plasmid copy number between the *E. coli* cells transformed with pET28b-TAT-EGFP1 (*left lane*) and pET28b-EGFP2 (*right lane*) respectively; **b** histogram showing the image analysis result

Discussion

In this study, we demonstrated that the TAT core domain markedly promoted soluble expression of heterologous proteins in *E. coli* and was not due to different amounts of transformed plasmid DNA. Subcellular localization studies revealed that the TAT-tagged protein was mainly expressed in the cytoplasm and also increased in the periplasmic space along with the time of expression, while the TAT-free protein was mainly distributed in the periplasm and only a minor amount in the cytoplasm. In order to further confirm our results, we have also constructed two pairs of prokaryotic expression vectors with different constructs, pET28b-neuroglobin versus pET28b-TAT-neuroglobin and pET28b-neuroglobin-EGFP versus pET28b-TAT-neuroglobin-EGFP with the same protocols. As expected, both the proteins, TAT-neuroglobin and TAT-neuroglobin-EGFP, displayed the same results of significant higher expression levels compared to TAT-free proteins, neuroglobin and neuroglobin-EGFP (data not shown).

E. coli has been widely used for heterologous proteins expression (Blackwell and Horgan 1991; Burton et al. 1991). It contained two important subcellular structures, the cytoplasm and periplasm, for the expression of heterologous proteins. Some studies revealed that large amounts of expressed heterologous proteins in the cytoplasm tended to form inclusion body because of protein misfolding (Schein 1991). The periplasm was an oxidizing environment, which contained a variety of enzymes and molecular chaperones, and benefited for correct folding of exogenous proteins as previous reports (Baecker et al. 1978; Vazquez-Laslop et al. 2001; Ewis and Lu 2005). However, our current studies demonstrated that the TAT core domain markedly increased the yield of heterologous proteins and

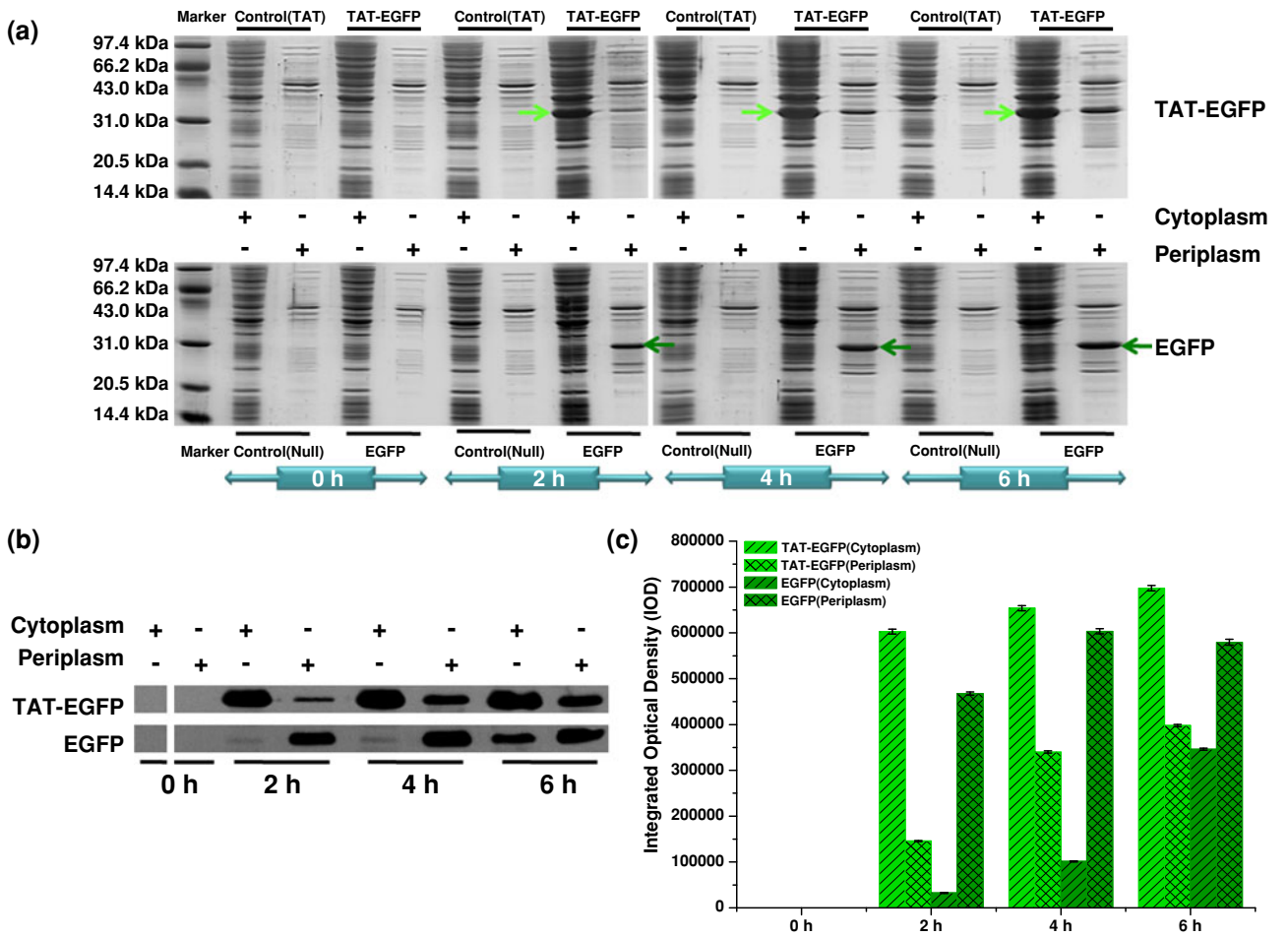


Fig. 6 Subcellular localization of the TAT-tagged and TAT-free proteins expressed in *E. coli* cells. **a** SDS-PAGE analysis of the cytoplasmic and periplasmic components of TAT-EGFP and EGFP; **b** western blot analysis of the cytoplasmic and periplasmic components of TAT-EGFP and EGFP; **c** histogram analysis the subcellular localization of TAT-EGFP and EGFP. 'Control (Null)' indicated the empty vector of pET28b. 'Control (TAT)' indicated the empty vector of pET28b-TAT. Arrowheads indicated TAT-EGFP or EGFP

also increased their solubility both in cytoplasm and periplasm, suggesting an alternative and valuable choice for promoting heterologous protein expression in *E. coli*. Our data also suggested that the TAT-free protein was mainly expressed in the periplasm, but the mechanism was still unknown, although probably depending on some other protein transport systems.

Although many PTDs have been found, the HIV-1-coded TAT peptide is the most widely used transporter for heterologous proteins traversing biomembranes. Frankel and Green demonstrated independently that the purified full-length TAT protein (86 amino acids) could transduce mammalian cells when added to the medium (Frankel and Pabo 1988; Green and Loewenstein 1988). Further studies revealed that the hendecapeptide basic domain TAT 47-57 (YGRKKRRQRRR) was the core functional domain for delivering heterologous macromolecules across biomembranes (Lindsay 2002; Wadia and Dowdy 2003; Snyder and Dowdy 2004). The basic domain of the TAT peptide is one of the shortest known cell-penetrating peptides with major accumulation in the nucleus, especially in the nucleolar region because it contains the nuclear localization signal (NLS) GRKKR (Vives et al. 1997). Some studies have revealed that basic amino acids have important roles in the PTD-mediated protein transduction process (Fawell et al. 1994; Vives et al. 1997; Prochiantz 2000; Jin et al. 2001). Anyway, the TAT peptide-induced penetrating approach seemed to be an valuable choice for transducing a wide variety of cargos across biomembranes and into intact tissues including the brain. For example, the TAT peptide is able to carry heterologous proteins across the blood/brain barrier, striatum, and midbrain, suggesting a potential value for developing novel therapeutic strategies for nervous system diseases (Wu et al. 2006).

Despite the ability of HIV-1 TAT peptide to deliver heterologous proteins across biomembranes has been

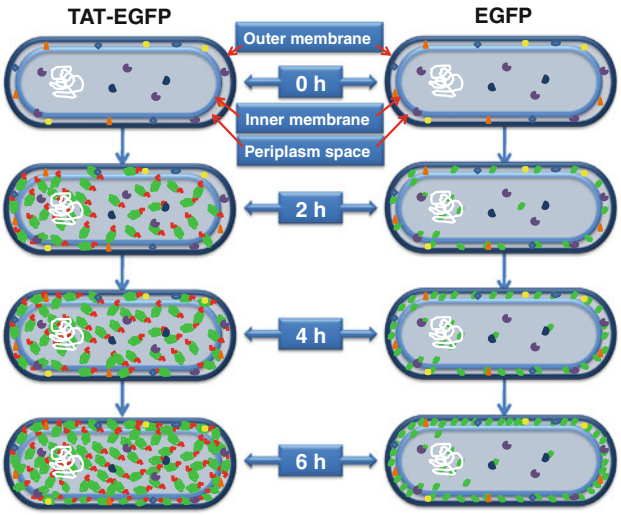


Fig. 7 Schematic diagram of the hypothesis model on TAT-promoted expression of heterologous proteins. *Left panel*: model of TAT-EGFP transportation process; *Right panel*: model of EGFP transportation process. Arrowheads indicated the outer membrane, inner membrane and periplasm space of the *E. coli* cells respectively

widely reported (Nagahara et al. 1998; Schwarze et al. 1999; Vocero-Akbani et al. 1999; Kwon et al. 2000; Caron et al. 2001; Jin et al. 2001; Wills et al. 2001; Ryu et al. 2003; Ryu et al. 2004; Wu et al. 2006), there is no study on whether the TAT peptide can promote the expression of fusion proteins. Recently, many prokaryotic expression vectors for heterologous proteins expression have been developed; however, the low yield and solubility of these heterologous proteins remain major limitations. Our current studies have demonstrated that the TAT peptide can increase both the yield and the solubility of heterologous proteins with complete biological activity. Accordingly, protein expression coupled with the TAT peptide is probably a novel breakthrough for heterologous protein expression, and might provide new opportunities for protein engineering.

Conclusion

Our data showed that the TAT peptide markedly promoted the soluble expression of heterologous proteins in *E. coli* with an known mechanism, at least not due to different amounts of transformed plasmid DNA. The expressed TAT-tagged protein was mainly located in cytoplasm and also increased in the periplasmic space along with the time of expression. The TAT peptide-coupled protein expression technique is a novel approach for promoting the study of protein engineering.

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