Research Paper

Insertion of the Designed Helical Linker Led to Increased Expression of Tf-Based Fusion Proteins

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Purpose. To demonstrate a high-level expression of transferrin (Tf)-based fusion proteins by inserting a helical linker between two protein domains.

Methods. Tf-based fusion proteins were designed to contain oligonucleotides encoding a helical linker inserted between the protein domains. Plasmid constructs were transfected into HEK293 cells and the secreted fusion proteins were purified from conditioned serum free media. Expression was assessed using both SDS-PAGE and Western Blot using anti-hGH, G-CSF, or Tf antibodies; protein bands were analyzed using Quantity One software. The function of fusion proteins consisting of human growth hormone (hGH) and Tf was evaluated in Nb2 cell proliferation assays.

Results. The fusion proteins containing a helical linker, hGH-$(H4)_2$-Tf and Tf-$(H4)_2$-hGH, were expressed 1.7-and 2.4-fold higher, respectively, with a twofold lower ED_{50} than the hGH-Tf fusion protein without a helical linker. The Tf-$(H4)_2$-G-CSF fusion protein exhibited a greater expression with an 11.2-fold increase compared with Tf-G-CSF fusion protein.

Conclusions. The helical linker introduced in Tf-fusion proteins resulted in a high-level of expression with improved *in vitro* bioactivity. This approach provides a simple method to increase poor expression of other fusion proteins.

KEY WORDS: domain switch; fusion protein; G-CSF; helical linker; hGH; increased-expression; transferrin.

INTRODUCTION

High quantities of recombinant proteins ranging from hundreds of milligrams to grams must be produced in order to carry out preclinical evaluations and clinical trials (1–3). Unfortunately, the potential therapeutic proteins with poor expression face an obstacle to make it through clinical trials to final approval by the FDA. Protein therapeutics developed from recombinant hormones, growth factors and cytokines

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ABBREVIATIONS: $(H4)_2$, 2 copies of helical linker [A(EAAAK)$_4$A]$_2$; G-CSF, granulocyte colony stimulating factor; G-CSF-Tf, granulocyte colony stimulating factor-transferrin recombinant fusion protein; hGH, human growth hormone; hGH-$(H4)_2$-Tf, hGH-Tf fusion protein with two copies of helical linker; hGH-Tf, human growth hormone-transferrin recombinant fusion protein; H4, 1 copy of helical linker A(EAAAK)$_4$A; Tf, human serum transferrin; Tf-G-CSF, transferrin-granulocyte colony stimulating factor recombinant fusion protein with Tf at N-terminus; Tf-hGH, transferrin-human growth hormone fusion protein with Tf at N-terminus; Tf-r(H4)$_2$-G-CSF, Tf-G-CSF fusion protein with a reversed (H4)$_2$ DNA sequence inserted. Nb2 cells, rat T lymphoma cells that proliferate upon stimulation by the bioactive hGH; Tf-$(H4)_2$-G-CSF, Tf-G-CSF fusion protein with 2 copies of helical linker; Tf-$(H4)_2$-hGH, Tf-hGH fusion protein with 2 copies of helical linker.

express at relatively low levels, not only increasing the manufacturing cost but also delaying further product evaluation. Some successful protein therapeutics are recombinant fusion proteins consisting of cytokines or growth factors fused with the Fc portion of IgG1 or immunotoxin and are expressed as single polypeptides with dual biological activities (4,5). These therapeutic fusion proteins, including Enbrel® (TNF-R/Fc-IgG1), Ontak® (IL-2/diphtheria toxin), Orencia® (CTLA-4/Fc-IgG1) and Amevive® (LFA-3/Fc-IgG1) (6), may experience poor expression as the fusion partners interfere with each other for optimal translation, especially in mammalian cells. Since mammalian cells are the preferred choice for producing some therapeutic proteins, as posttranslational modifications in these cells may be associated with reduced immunogenicity compared to other systems (3), a simple strategy that enhances the expression of therapeutic fusion proteins in mammalian cells would be desirable.

Typically, the problem of low expression is improved by incorporating carbohydrate-binding module (CBM) and maltose-binding protein (MBP) as fusion partners to the target protein (7,8). However, these fusion partners are generally removed during or after purification by introducing peptide linkers with cleavage sites for endopeptidases such as thrombin and factor Xa (8). Conceivably, this approach is not feasible for large-scale production of target proteins because it requires numerous steps of column purification and enzymatic processing, limiting the production capacity and possibly causing non-specific cleavage.

The selection of a peptide linker with the ability to maintain domain function of the fusion protein is becoming important (9–12). Recently, we designed a helical linker with 50 amino acids using an EAAAK helix-forming motif based on a previous study (10), and inserted the linker between granulocyte colony stimulating factor (G-CSF) and Tf moieties, leading to increased biological activity (13). Most recently, we found that the insertion of the same helical linker in Tf-fusion proteins resulted in a high-level expression in HEK293 cells as compared to the same fusion proteins without the helical linker. Here we report the helical linker-dependent increase of expression in two Tf-based fusion proteins, G-CSF and human growth hormone (hGH), and provide evidence of a high-level of expression for both proteins regardless the level of original expression without the linker. Conceivably, this approach can be introduced and applied to other fusion proteins with limited to no expression, greatly improving the production yield for downstream applications.

MATERIALS AND METHODS

Preparing Gene Fusion Constructs in pcDNA3.1(+)

Fusion constructs for Tf-based fusion proteins containing either hGH or human G-CSF were designed and established in mammalian expression vector pcDNA3.1(+) (Invitrogen) based on a previous report (14). Briefly, the DNA sequences encoding for hGH or G-CSF were subcloned and fused in frame to the sequences encoding for Tf. The DNA sequences encoding for the signal peptide from the N-terminus domain was incorporated in the polypeptide; however, the stop codon from the N-terminus domain was deleted for uninterrupted translation. The final constructs were verified by DNA sequence analysis.

Helical Linker Insertion

Two domains between Tf and hGH or G-CSF in the fusion protein were fused by leucine (L) and glutamic acid (E), a product of *Xho*I restriction site. The helical linker, H4 and (H4)$_2$, LEA(EAAAK)$_4$ALE and LEA(EAAAK)$_4$ALEA(EAAAK)$_4$ALE, respectively, were prepared and inserted according to the previous study (13). The orientation, sequences and copy numbers of the helical linker were confirmed by DNA sequence analysis.

Production of Fusion Protein

The human embryonic kidney 293 cells (HEK293 or HEK293T; ATCC) were cultured in DMEM media (Mediatech) containing 10% FBS, 50 units penicillin/50 μg streptomycin in a humidified incubator at 37°C with 5% CO_2. HEK293 cells were seeded at near confluence in six-well plates (Costar) and transiently transfected with 2 μg expression constructs and 5.5 μl Lipofectamine 2000 (Invitrogen). The transfected cells were allowed to express fusion proteins in serum free CD293 media (Invitrogen) for 5 days. The conditioned media containing the fusion protein was then harvested, clarified by centrifugation, and concentrated using Amicon Ultra-4 or Ultra-15 filtering devices (Millipore).

SDS-PAGE and Western Blots

The fusion proteins were fractionated on a 10% SDS-PAGE or 4–20% pre-cast gel (Thermo Scientific) and visualized by staining with Coomassie blue. For Western blot analysis, the fusion proteins were transferred to a PVDF membrane (GE healthcare) and blocked with 5% non-fat milk for 1 h at room temperature, after separating on SDS-PAGE. hGH and Tf were identified by using goat anti-hGH monoclonal antibody (1:1,000; R&D Systems) and goat anti-human Tf antibody (1:5,000) as primary antibodies and rabbit anti-goat antibody conjugated to HRP as secondary antibody. Likewise, the G-CSF was detected by using rabbit anti-human G-CSF (1:10,000) as primary antibody and donkey anti-rabbit antibody conjugated to HRP (1:10,000) as secondary antibody. All antibodies were obtained from Sigma, unless mentioned otherwise. ECL plus reagents (GE Healthcare) and ChemiDoc XBR (Bio-Rad) were used for developing and capturing the hGH-fusion proteins. X-ray film was used to develop G-CSF-fusion proteins. The expression of both hGH-and G-CSF-fusion proteins were analyzed using Quantity One software (Bio-Rad), and results from either anti-hGH or anti-G-CSF Western blots are comparable to that of anti-Tf Western blots.

In Vitro Cell Proliferation

Nb2 cells (Sigma) derived from rat T lymphoma cells were cultured as suspension in RPMI 1640 media (Mediatech) supplemented with 2 mM glutamine, 10% FBS, 10% horse serum (HS; Invitrogen), 50 units of penicillin/50 μg streptomycin, and 50 μM 2-mercaptoethanol (15) in a humidified incubator at 37°C with 5% CO_2. For proliferation assays, Nb2 cells were washed extensively in a serum free RPMI 1640 media, re-suspended in assay media that included 10% HS but not FBS, and counted with a Z1 Coulter particle counter (Beckman Coulter). About 5,000 Nb2 cells per well were seeded into 96-well plates in 200 μl assay media, starved for 24 h, treated with hGH or fusion protein whose dose was normalized to that of hGH, and incubated for 4 days. Next, the cells were added with 20 μl of Alamar Blue dye (Biosource) and incubated overnight for color development. The UV absorbance was measured at 570 nm using a Genios spectrophotometer (Tecan) and corrected by subtracting the control without treatment. The ED_{50} was defined as the dose of fusion protein that led to half of maximum proliferation.

RESULTS

Establishing Gene Fusion Constructs

To investigate whether the insertion of a helical linker between the protein domains in the Tf-based fusion protein improves the expression, we constructed three pairs of gene fusion plasmids with or without the inserted helical linker, and confirmed that the insertion, orientation and number of copies were correct. Subsequently, the plasmid constructs were transfected to HEK293 or HEK293T cells to produce fusion proteins.

Comparing hGH-Tf and hGH-(H4)$_2$-Tf Fusion Proteins for Expression

The hGH-Tf fusion protein consists of human growth hormone and Tf linked by a short di-peptide linker; whereas the hGH-(H4)$_2$-Tf fusion protein is linked by a helical linker with 50 amino acids. To assess the level of expression, the fusion proteins with or without the helical linker were analyzed by Western blot as well as SDS-PAGE with Coomassie stain. Both anti-hGH and anti-Tf Western blots detected a band corresponding to approximately 100 kDa, which is the sum of molecular weights from Tf (79 kDa) and hGH (22 kDa), and confirmed that the fusion protein was composed of two moieties including Tf and hGH (Fig. 2 lanes 3 and 4). To evaluate and compare the level of expression, band-densities for each fusion protein were quantified and analyzed. The density data revealed that the fusion protein with the helical linker expressed at a level 1.7 fold higher than the original fusion protein without the helical linker (Table I, Fig. 1 lanes 3 and 4).

In addition, fusion proteins were analyzed by SDS-PAGE stained with Coomassie blue to confirm results from the band-density analysis from Western blots, and to evaluate relative purity and abundance. The results from SDS-PAGE demonstrated that the fusion proteins were expressed with high purity (~90%) and high abundance (~95%) at a molecular weight of 100 kDa (Fig. 3 lane 5). Furthermore, band-density analysis from Coomassie blue-stained SDS-PAGE showed that the expression of fusion protein with the helical linker was about 1.7 fold higher than the original fusion protein without the helical linker (Fig. 3 lane 4), confirming results from Western blot analysis.

Comparing Tf-hGH and Tf-(H4)$_2$-hGH Fusion Proteins for Expression

Thus far, the fusion proteins evaluated were oriented to have a N-terminal hGH domain and C-terminal Tf domain. To investigate the effect of the helical linker on the expression of fusion proteins with different orientation, the Tf-and hGH-domains were switched to produce two new fusion proteins: Tf-hGH and Tf-(H4)$_2$-hGH. The two fusion proteins were expressed and the yield of expression was compared. The Western blot data with fusion protein specific antibodies including anti-hGH and anti-Tf confirmed the identity and molecular weight (100 kDa) of the fusion protein, as well as the insertion of helical linker as shown by

slightly increased molecular weight (Fig. 1 lanes 5 and 6, Fig. 2 lanes 5 and 6). The band-density analysis from Western blot data showed the fusion protein with the inserted helical linker expressed at a higher level with a ~2.4 fold increase as compared to the original fusion protein without the helical linker (Table I). The SDS-PAGE with Coomassie stain revealed that both fusion proteins were expressed with high purity (~90%) and high abundance (90%) but the fusion protein with the helical linker expressed at high levels with a ~2.4 fold increase compared to the original fusion protein without the helical linker (Fig. 3 lane 7 and 6, respectively), further confirming the Western blot data.

Comparing Tf-G-CSF and Tf-(H4)$_2$-G-CSF Fusion Proteins for Expression

To study the broader implication of the helical linker insertion on the increased expression of Tf-based fusion proteins, Tf-G-CSF fusion protein consisting of transferrin and granulocyte colony-stimulating factor was constructed, and subsequently, the helical linker was inserted. These two fusion proteins were expressed to compare the level of expression. The Western blot using anti-G-CSF antibody detected a 100-kDa band equivalent to the molecular weight of Tf-G-CSF fusion protein (~80+19 kDa), confirming the identity and molecular weight (Fig. 4). Moreover, the Western blot data revealed that the expression from the Tf-G-CSF fusion protein was too low to detect convincingly (Fig. 4 lane 3). In contrast, the Tf-(H4)-G-CSF and Tf-(H4)$_2$-G-CSF fusion proteins expressed at elevated levels with a 7.8 and an 11.2 fold increase as compared to original fusion protein without the linker, respectively, providing notable evidence for the improved expression of Tf-based fusion proteins by the insertion of a helical linker (Fig. 4 lanes 4 and 5, Table I). The fusion protein expressed with a reversed coding sequence for the helical linker (Tf-r(H4)2-G-CSF) failed to express (Fig. 4 lane 6). Taken together, the helical linker insertion between Tf and G-CSF domains led to a significant increase in expression, comparing with the original fusion protein without the helical linker.

Comparing In Vitro Cell Proliferation

The hGH-(H4)$_2$-Tf and Tf-(H4)$_2$-hGH fusion proteins were tested in vitro to determine whether they induce Nb2 cell proliferation. The Nb2 cells treated with either hGH-(H4)$_2$-Tf or Tf-(H4)$_2$-hGH fusion protein demonstrated higher prolifer-

Table I. The Ratio of Expression of Tf-fusion Proteins with or without Helical Linker

| Linker (x) | G-CSF Fusion Proteins | | hGH Fusion Proteins | |
	Tf-x-G-CSF	G-CSF-x-Tf	Tf-x-hGH	hGH-x-Tf
No linker	1	1	1	1
H$_4$	7.8	ND	ND	ND
(H$_4$)$_2$	11.2	1.44	2.39	1.66

x linker inserted between Tf and hGH or G-CSF, (H4) helical linker, (H4)$_2$ two copies of helical linker, no linker two domains were fused by LE peptide, ND not determined
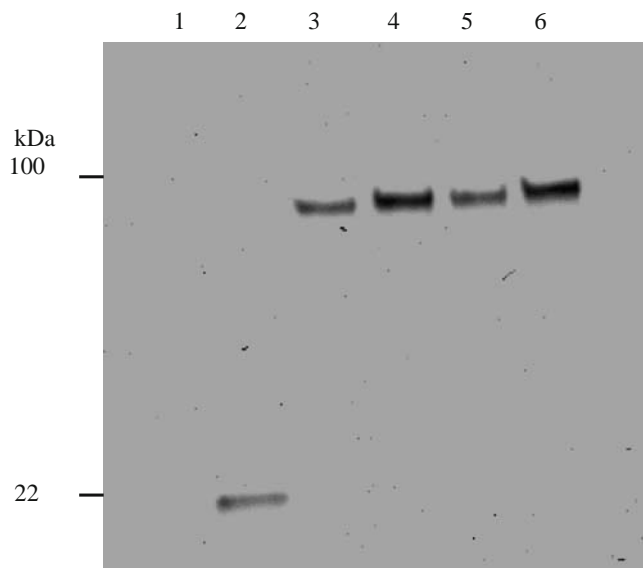
Fig. 1. High-level expression by the insertion of helical linker in both hGH-Tf and Tf-hGH fusion proteins as analyzed by Anti-hGH Western blot. Four fusion proteins with or without the inserted helical linker, expressed in serum free media, were analyzed by Western blot using goat anti-hGH monoclonal antibody (1:1,000). The signal was detected using HRP-conjugated rabbit anti-goat secondary antibody (1:1,000) and ECL reagents. The image was recorded and analyzed by ChemiDoc XBR (Bio-Rad). *Lane 1*: Tf (negative control); *lane 2*: hGH (10 ng); *lane 3*: hGH-Tf; *lane 4*: hGH-$(H4)_2$-Tf; *lane 5*: Tf-hGH; *lane 6*: Tf-$(H4)_2$-hGH.
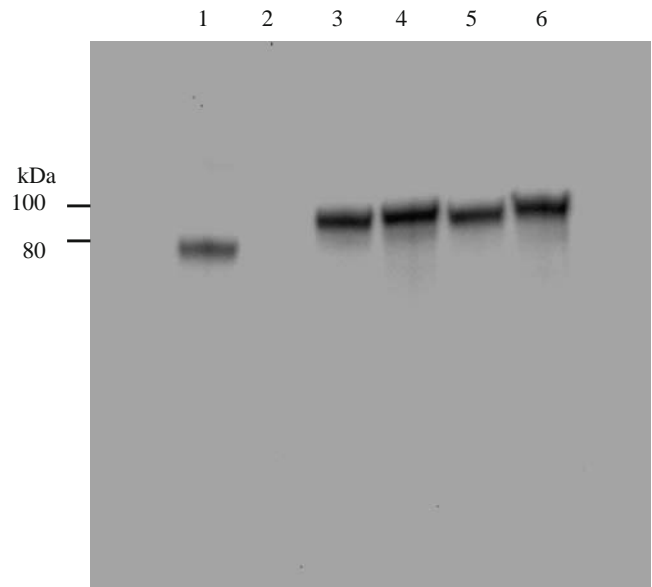

Fig. 2. High-level expression by the insertion of helical linker in both hGH-Tf and Tf-hGH fusion proteins as analyzed by Anti-Tf Western blot. Four fusion proteins with or without the inserted helical linker, expressed in serum free media, were analyzed by Western blot using goat anti-Tf antibody (1:5,000). The signal was detected using rabbit anti-goat secondary antibody conjugated to HRP (1:1,000) and ECL reagents. The image was recorded and analyzed by ChemiDoc XBR. *Lane 1*: Tf (50 ng); *lane 2*: hGH (negative control); *lane 3*: hGH-Tf; *lane 4*: hGH-$(H4)_2$-Tf; *lane 5*: Tf-hGH; *lane 6*: Tf-$(H4)_2$-hGH.

ation activity as compared to Nb2 cells treated with the hGH-Tf fusion protein without helical linker (Table II).

DISCUSSION

The success of constructing biologically active recombinant G-CSF-Tf fusion protein with the helical linker (13,14) led us to pursue the feasibility of producing other Tf fusion proteins. To achieve this goal, we constructed recombinant fusion protein consisting of Tf and hGH, and introduced two copies of a helical linker $(H4)_2$ as a spacer between two protein domains. As previously reported in G-CSF fusion protein (13), the insertion of the helical linker also increases the *in vitro* biological function of hGH fusion protein as shown in the Nb2 cell proliferation assay (Table II).

Besides the increase of *in vitro* biological activity, we found that the expression level of both fusion proteins with the helical linker, i.e., G-CSF-$(H4)_2$-Tf and hGH-$(H4)_2$-Tf, was significantly higher than that of fusion proteins without the linker. We further investigated the effect of helical linker insertion on the expression of fusion proteins with a Tf-domain switch, i.e., Tf-$(H4)_2$-G-CSF and Tf-$(H4)_2$-hGH, which created a fusion protein with a different orientation. As shown in Fig. 3 and 4, the expression of fusion protein with Tf at the amino-terminus is generally very poor. However, the insertion of the helical linker significantly increased the expression of both fusion proteins with Tf at the amino-terminus (Table I).

We have reported previously that the expression of G-CSF-Tf fusion protein failed when a flexible and non-helical linker, consisting of glycine and serine $(GSSSS)_3$, was inserted between Tf and G-CSF domains (13). Others have also found that the insertion of IgG hinge region as a flexible linker between Tf and nerve growth factor (NGF) domains was ineffective for the expression of this fusion protein (12). To further demonstrate the requirement of the helical structure of the linker peptide for the increase of the fusion protein expression, we reversed the DNA sequence coding for the helical peptide to produce a linker with an identical peptide length, but a non-helical structure. Our results showed that there was no expression of the fusion protein, Tf-r$(H4)_2$-G-CSF (Fig. 4 lane 6). These findings led us to the conclusion that the increase of the expression of Tf-based fusion protein
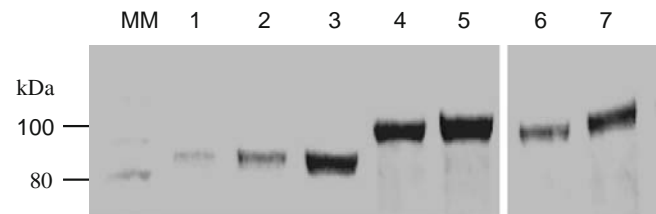

Fig. 3. Helical linker insertion led to high-level expression in both hGH-Tf and Tf-hGH fusion protein as analyzed by SDS-PAGE. Same volume of conditioned media (5 µl) from the transfected HEK-293 cells were fractionated using SDS-PAGE, stained with Coomassie blue, and analyzed with ChemiDoc XBR. *MM*: molecular weight marker; *lanes 1–3*: Tf control; *lane 4*: hGH-Tf; *lane 5*: hGH-$(H4)_2$-Tf; *lane 6*: Tf-hGH; *lane 7*: Tf-$(H4)_2$-hGH.
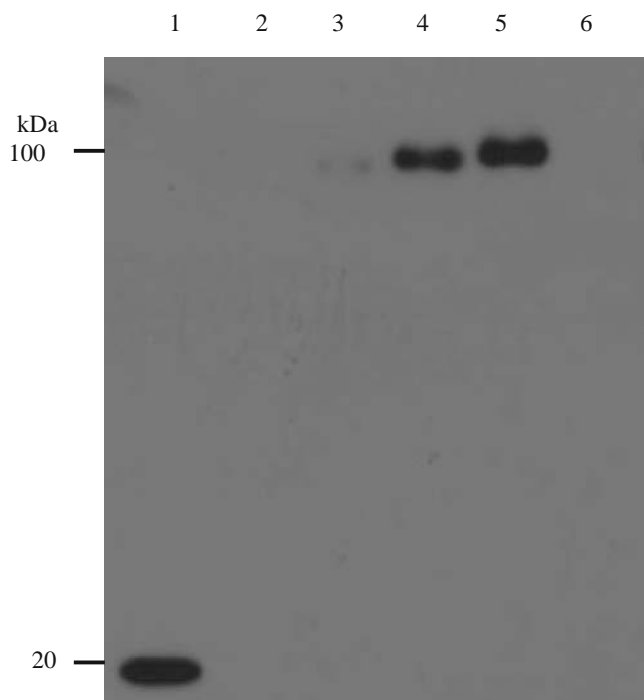
Fig. 4. Helical linker insertion led to high-level expression in both G-CSF-Tf and Tf-G-CSF fusion protein as analyzed by anti-G-CSF Western blot. Fifteen-microliter-conditioned media from the transfected HEK-293 cells were analyzed using anti-GCSF Western blot. *Lane 1*: G-CSF control; *lane 2*: pcDNA3.1(+) without the insert; *lane 3*: Tf-G-CSF; *lane 4*: Tf-(H4)-G-CSF; *lane 5*: Tf-(H4)$_2$-G-CSF; *lane 6*: Tf-r(H4)$_2$-G-CSF (fusion protein with a reversed (H4)$_2$ DNA sequence inserted, thus a non-helical linker in the product).

domains were important in controlling the rate of folding, unfolding, and stability of chimeric protein. It is likely that a large molecule, such as Tf-G-CSF or Tf-hGH fusion protein with a molecular weight of 100 kDa, requires large conformational space to fold correctly. The stable and efficient folding may drive the equilibrium towards an increased expression and accumulation of the fusion protein. Conceivably, the linker with a helical structure can hold the domains at a distance, providing a larger space for correct folding.

Another possible reason for the increased expression is that a helical linker with its secondary structure may be resistant to enzymatic cleavage by protecting the target amino acids from protease recognition, thereby increasing overall stability of the fusion protein (17). Further studies that evaluate the stability of the helical linker against common proteolytic enzymes including trypsin and chymotrypsin, would aid partially in our understanding of the mechanisms responsible for the increased expression.

In general, a high level expression of fusion proteins may be achieved: (1) by designing expression vectors with strong promoters and episomal origins; (2) by transfecting host cells such as HEK293T and HEK293 EBNA1 with expression of episomal antigens including SV40 large-T antigen and EBV nuclear antigen, respectively; (3) by selecting transfection reagents with highest gene transfer efficiency; and (4) by developing or using culture media with enhanced cell proliferation and survival but with minimum cell death and apoptosis (1–3). Furthermore, the addition of sodium butyrate and peptone into the culture media containing the transfected cells can have positive effect on the expression of target protein. However, long-term consequences of using such components in culture media remain controversial and unknown (18,19). Results in this report demonstrate that a high level expression of Tf-fusion proteins can be achieved by the insertion of a helical peptide linker between the two protein domains. If the increase of expression can be demonstrated in fusion proteins other than Tf, it will provide a simple and practical technique to improve the production of recombinant fusion proteins for therapeutic and diagnostic uses.

CONCLUSIONS

We found that the insertion of a helical peptide as the linker in Tf-based fusion proteins led to a high level of expression, with superior *in vitro* bioactivity. Given the straightforward approach and ease of both designing and introducing the helical linker in the fusion protein, this qualifies as a feasible strategy for the production of therapeutic fusion proteins at high levels in mammalian cells.

is due to the non-flexible and helical nature of the linker that is inserted between the two protein domains.

The exact mechanism responsible for this increased expression is unclear. However, the rigid, extended nature, as well as the composition, of the helical linker may help increase the rate of hGH-and Tf-domain folding. A proper folding will enhance the stability of the newly translated polypeptide and, consequently, will increase the expression of Tf fusion proteins in the conditioned media. By using fluorescent resonance energy transfer (FRET) technique, Arai *et al.* found that the insertion of a helical linker between the enhanced green fluorescent protein (EGFP) domain and enhanced blue fluorescent protein (EBFP) domain in a chimeric protein increased the distance and kept two domains apart (10). Furthermore, Robinson and Sauer (16) reported that the composition and length of linker between two

Table II. Comparing Nb2 Cell Proliferation Activity of hGH-Tf Fusion Protein, with or without Helical Linker

Linker (x)	ED$_{50}$ (ng/mL)		
	hGH	hGH-x-Tf	Tf-x-hGH
No linker	0.25	1.85	NA[a]
(H4)$_2$	–	0.85	0.80

[a] Was not determined due to low yield of the fusion protein

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