Effects of overexpression of X-box binding protein 1 on recombinant protein production in Chinese hamster ovary and NS0 myeloma cells

Sebastian C. Y. Ku¹³, Daphne T. W. Ng¹, Miranda G. S. Yap¹²³, Sheng-Hao Chao¹∗

¹Bioprocessing Technology Institute, Biomedical Sciences Institutes, 20 Biopolis Way, #06-01, Singapore 138668
²Department of Chemical & Biomolecular Engineering, National University of Singapore, 10 Kent Ridge Crescent, Singapore 119220
³NUS Graduate School for Integrative Sciences and Engineering, National University of Singapore, 10 Kent Ridge Crescent, Singapore 119220

Keywords: CHO cells; erythropoietin; recombinant protein production; unfolded protein response; XBP-

∗corresponding author

E-mail: jimmy_chao@bti.a-star.edu.sg
Tel: +65 6478 8899
Fax: +65 6478 9561

© 2007 Wiley Periodicals, Inc.
Received January 8, 2007; Accepted June 18, 2007
ABSTRACT

X-box binding protein 1 (XBP-1) is a key regulator of the cellular secretory pathway and unfolded protein response. It has been shown that the spliced form of XBP-1, XBP-1S, functions as a transcription activator and up-regulates many genes associated with protein secretion and biosynthesis of endoplasmic reticula. Since the production of some recombinant proteins is widely believed to be limited by the secretory capacity of the host cell, an increase in protein production may be achieved by overexpressing XBP-1S. In this study, the effects of XBP-1S on the productivity of monoclonal antibody, interferon γ, and erythropoietin are examined in Chinese hamster ovary (CHO) and NS0 cell lines. Results show that XBP-1S may become a determinative factor only when accumulation of recombinant proteins exceeds the secretory capacity of the host cell. In transient transfection systems where a bottleneck in protein secretion was achieved, overexpression of XBP-1S improved protein titers by up to 2.5-fold. In contrast, overexpression of XBP-1S had no detectable effects on protein productivity of stable cell lines that did not exhibit any secretory bottleneck. We conclude that overexpression of XBP-1S is an effective strategy in enhancing recombinant protein production when the secretory pathway of the host cell is saturated by high-level synthesis of recombinant proteins.
INTRODUCTION

Most of the biopharmaceutical products like monoclonal antibody (MAb), interferon γ (IFNγ), and erythropoietin (EPO), are secreted proteins. As the endoplasmic reticulum (ER) plays a quintessential role in the secretory pathway, several studies have been done on the interplay between the production of secreted recombinant proteins and the regulation of ER proteins in the host cells. For example, elevated expression of a number of recombinant proteins in Chinese hamster ovary (CHO) cells by sodium butyrate treatment was found to induce expression of two glucose-regulated proteins (GRPs), GRP78/BiP and GRP94, both ER-resident chaperones involved in protein folding and assembly (Dorner et al., 1989). Proteomic analysis of antibody-producing NS0 myeloma cells with varying productivity revealed a positive correlation between specific antibody productivity and expression levels of ER chaperones like BiP and endoplasm, and protein disulfide isomerase (PDI) (Smales et al., 2004). Similarly, a human embryonic kidney cell line, HEK-293, expressing high levels of transferrin was found to have elevated expression of BiP (Jones et al., 2005). These observations demonstrate that high-level production of recombinant proteins associates with up-regulation of the ER proteins involved in protein secretion. It follows that host cell engineering strategies targeting the secretory pathway may serve to enhance recombinant protein production.

To date, the most common approach in secretion engineering has been one involving overexpression of ER chaperones like BiP and PDI. While such a strategy increases heterologous protein production in yeast (Shusta et al., 1998; Smith et al., 2004) and insect cells (Ailor et al., 1998; Hsu et al., 1997), it has not consistently achieved the
same effects in mammalian cells (Borth et al., 2005; Davis et al., 2000; Kitchin et al., 1995). Surprisingly, reduction of the level of endogenous BiP, rather than its overexpression, was found to improve secretion of tissue plasminogen activator in CHO cells (Dorner et al., 1988). Given the complexity of mammalian gene regulation, targeting single components of the secretory pathway in isolation may not always produce the desired outcome. As such, a strategy that can modulate the secretory machinery in a global fashion is needed. To accomplish this, X-box binding protein 1 (XBP-1), a key regulator for the secretory pathway, may be a more effective gene target compared to particular ER chaperones.

XBP-1 is a basic leucine zipper transcription factor that plays a major role in the cellular unfolded protein response (UPR), which is triggered by intracellular accumulation of unfolded or malfolded proteins. There are two protein isoforms of XBP-1, XBP-1U and XBP-1S. XBP-1U is translated from the unspliced mRNA of XBP-1. It is transcriptionally inactive and is the dominant isoform under non-stress conditions. Accumulation of unfolded or malfolded proteins in the ER induces UPR and activates the endoribonuclease activity of IRE1, a ER transmembrane protein, which induces the excision of 26 bases from the XBP-1 transcript (Mori, 2003; Yoshida et al., 2001). The spliced mRNA translates into a longer and transcriptionally active protein, XBP-1S. XBP-1S activates expression of many ER chaperones and results in increased contents of ER, golgi, and mitochondria (Lee et al., 2003). XBP-1S plays an essential role in the up-regulation of the secretory capacity of plasma cell to prepare it for high level secretion of immunoglobulins (Iwakoshi et al., 2003b; Iwakoshi et al., 2003a; Shaffer et al., 2004). Overexpression of XBP-1S in primary B cells enhanced immunoglobulin M (IgM)
production whereas IgM production was significantly decreased in XBP-1 knock out cells (Iwakoshi et al., 2003b), clearly demonstrating the importance of XBP-1S in the secretory pathway of B cells.

Given its role as a global regulator of the secretory pathway, XBP-1S may be an effective target for host cell engineering to improve production of secreted recombinant proteins. Indeed, Tigges et al. reported that overexpression of XBP-1S improved production of human placental secreted alkaline phosphatase (SEAP), *Bacillus stearothermophilus*-derived α-amylase, and human vascular endothelial growth factor 121 in CHO-K1 cells by expanding the secretory capacity of the cells (Tigges et al., 2006). Our current study was conducted with two primary objectives: (i) to evaluate the effects of XBP-1S overexpression on the production of MAb, IFNγ, and EPO; and (ii) to establish a qualitative correlation between recombinant protein expression level and titer improvement caused by XBP-1S overexpression. To validate previously reported data by Tigges et al. (Tigges et al., 2006), XBP-1S was overexpressed in three CHO stable cell lines producing MAb, IFNγ, and EPO, respectively, and the resulting protein titer was analyzed. To investigate the correlation between expression levels of recombinant proteins and overexpression of XBP-1S, transient EPO production systems in CHO-K1 and NS0 cells with a wide range of productivities were established by titration of the EPO expression plasmid. Over this productivity range, EPO titer in cultures with and without XBP-1S co-expression was analyzed and compared. The conclusions drawn from these analyses provide novel insights into the effectiveness of XBP-1S targeting as a means to improve recombinant protein production.
MATERIALS AND METHODS

Vector Construction

To construct the vector CMV-mXBP-1U, the cDNA encoding unspliced murine XBP-1 (cDNA clone MGC:36774, IMAGE:2648085; Open Biosystems) was generated by site-directed mutagenesis of two base pairs located in the 26 nt splice intron of the full-length XBP-1 cDNA as previously described (Iwakoshi et al., 2003b). In short, two guanine residues at positions 487 and 490 (GenBank accession number BC029197) were mutated to adenine without changing the amino acid sequence. The resulting cDNA cannot undergo alternative splicing and will hence express only XBP-1U (Yoshida et al., 2001). The open reading frame of XBP-1U was then cloned into pcDNA3.1(+) (Invitrogen), a mammalian expression vector containing the cytomegalovirus (CMV) major immediate early (MIE) promoter, between Hind III and Xho I sites. To construct the vector CMV-mXBP-1S, the cDNA encoding spliced murine XBP-1 was amplified from total RNAs of a murine hybridoma, CRL1606 (American Type Culture Collection, ATCC) by RT-PCR. The cDNA was then cloned into pcDNA3.1(+) between Nhe I and Afl II sites. Human EPO cDNA was amplified from a human leukocyte cDNA library (Clontech) and cloned into pcDNA3.1(+) between BamH I and EcoR V sites, creating the vector CMV-EPO. The vector CMV-GFP was generated by amplification of the cDNA for green fluorescent protein (GFP) from pEGFP-C2 (Clontech) and cloning into pcDNA3.1(+) using EcoR V and Xba I sites. The vectors BiP-Luc and ATF6(1-373) were provided by Dr. Kazutoshi Mori (Yoshida et al., 2000).
**Cell Culture**

CHO-K1 cells (ATCC) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS; Invitrogen). A CHO cell line stably expressing EPO, CHO-EPO, was generated by transfecting CHO-K1 cells with CMV-EPO and selecting with Geneticin (G418; Invitrogen). Generation of a CHO cell line stably expressing human IFNγ, CHO-IFNγ, was previously described (Scahill et al., 1983). CHO-EPO cells were cultured in DMEM supplemented with 10% FBS and 800 μg/ml G418 and CHO-IFNγ cells were cultured in DMEM supplemented with 10% FBS and 250nM methotrexate (MTX; Sigma). NS0 cells (European Collection of Animal Cell Cultures) were cultured in shake flasks in DMEM supplemented with 10% FBS. A CHO cell line stably expressing a human monoclonal antibody, CHO-MAb, was generated by transfecting CHO-DG44 cells with plasmids encoding the light and heavy chains, selected with G418, and amplified in 250 nM MTX. The amplified pool of CHO-MAb cells were cultivated as suspension cultures in 50% CD CHO (Invitrogen) and 50% HyQ® PF-CHO™ (Hyclone) media, supplemented with 6 mM L-glutamine (Sigma), 600 μg/ml G418, 250 nM MTX, and 0.1% Pluronic® (Invitrogen).

**ELISA and Western Blotting Analysis**

Protein titers in culture supernatant were analyzed using ELISA kits for EPO (Roche) and IFNγ (HyCult Biotechnology) according to the manufacturers’ protocols. The reporting unit for EPO quantity is IU, in accordance to the unit of EPO standards supplied in the ELISA kit. For ease of comparison with other production systems, specific EPO productivity is converted from IU/10^6 cells/day to pg/cell/day using a
conversion factor of 140,000 IU/mg (Sun et al., 2006). For MAb ELISA, 96-well plates were pre-coated with 10 μg/ml of a goat anti-human IgG, IgA, and IgM antibody (SPD Scientific) and blocked in phosphate buffered saline containing 3% bovine serum albumin. The plate was then incubated with diluted samples of supernatant. After washing, an alkaline phosphatase-conjugated, Fc-specific antibody (Sigma) was added for further incubation. MAb concentration in each sample was then measured by adding SIGMAFAST™ p-nitrophenyl phosphate substrate (Sigma) for enzyme reaction. The standard curve for the MAb was generated by serial dilution of a purified human IgG (Sigma).

For Western blotting analysis, culture supernatant was electrophoresed and transferred onto a polyvinylidene difluoride membrane (Bio-Rad), which was then probed with a mouse monoclonal anti-EPO antibody purified from culture supernatant of a murine hybridoma HB-8209 (ATCC). Horseradish peroxidase conjugated anti-mouse IgG (Pierce) was used as secondary antibody. The blots were then incubated with SuperSignal West Pico Substrate (Pierce) and the chemiluminescent signal was detected using an X-ray film (Roche). The film was then scanned and band volume was quantitated using the GS-800 densitometer (Bio-Rad).

**Transient Transfection and Luciferase Assay**

Transfection of CHO-K1, CHO-EPO, and CHO-IFNγ cells was performed using the transfection reagent FuGENE 6 (Roche) according to the manufacturer’s protocol. Transfection efficiency was estimated by transfecting the cells with the CMV-GFP vector. About 60-70% of the transfected cells expressed GFP 24 hours post-transfection.
Unless otherwise mentioned, transient expression experiments were done in duplicates on 96-well plates. Supernatant from duplicate wells was collected and pooled 2 days post-transfection for enzyme-linked immunosorbent assay (ELISA) and Western blotting analyses.

Electroporation was performed using the Amaxa Nucleofector device (Amaxa) according to the manufacturer’s protocol. Nucleofector Kits V and C (Amaxa) were utilized for electroporation of CHO-MAb and NS0 cells, respectively. For CHO-MAb cells, electroporation was performed with $1 \times 10^6$ cells and 5 μg of blank vector or CMV-mXBP-1S expression plasmid. For NS0 cells, $2 \times 10^6$ cells and 3 μg of total plasmid were used. Cell numbers were counted 2-3 hours post-electroporation to determine the number of surviving cells. Using a GFP reporter plasmid, 60-80% transfection efficiency was estimated based on the expression of GFP 24 hours post-electroporation. Protein titers (MAb or EPO) were determined by ELISA.

Luciferase reporter assays were performed in 96-well format. 8,000 cells were seeded into each well one day prior to transfection using FuGENE 6 (Roche). Amount of plasmid DNA used for each transfection was 0.15 μg, which includes 0.05 μg BiP-Luc plasmid and 0.1 μg of an expression plasmid, ATF6α(1-373), mXBP-1S or mXBP-1U. The blank vector (0.1 μg) was used as a negative control. Two days post-transfection, luciferase activity was assayed using BrightGlo™ Luciferase Assay Kit (Promega) according to the manufacturer’s protocol. Luciferase signals were measured using the Genios microplate reader (Tecan).

**Quantitative Real-Time PCR**
The mRNAs in transfected cells were quantitated using quantitative real-time polymerase chain reaction (qRT-PCR) performed on the ABI PRISM® 7000 Sequence Detection System (Applied Biosystems). To prepare the template for qRT-PCR, total RNAs were extracted using the RNeasy Mini Kit (Qiagen), followed by reverse transcription using the Improm-II™ Reverse Transcription System (Promega) according to the manufacturers’ protocols. The resulting first strand cDNA was then used for qRT-PCR using Power SYBR® Green PCR Master Mix (Applied Biosystems) with the following primers: ERdj4 - (forward) 5’-CACTTCCAGACACACCAGGA-3’ and (reverse) 5’-TTCTCCTCTGCTGAGTGACA-3’; EPO - (forward) 5’-GCAGCAGCCGTAGAGGTC-3’ and (reverse) 5’-GGAGGGAGATGGCTTCCT-3’; Actin – (forward) 5’-AGCTGAGAGGGAAATTGTGCG-3’ and (reverse) 5’-GCAACGGAAACGCTCATT-3’. Actin transcript was used as a normalizing control for total RNA. Results were normalized by subtracting the threshold cycle number (C_T) of EPO to that of actin.
RESULTS

Overexpression of XBP-1S did not improve protein production in CHO stable cell lines

It was previously demonstrated that XBP-1S overexpression increased overall production of several recombinant proteins in CHO-K1 cells by expanding the secretory capacity of the cells (Tigges et al., 2006). To further confirm these observations, plasmids expressing murine XBP-1U and XBP-1S under the control of the CMV MIE promoter were constructed. The plasmid CMV-mXBP-1U contains two silent mutations that render the mRNA unspliceable, expressing only XBP-1U, whereas the plasmid CMV-mXBP-1S expresses only XBP-1S. Expression of these proteins was confirmed by Western blotting analysis of cell lysates prepared from the CHO-K1 cells transfected with CMV-mXBP-1U and CMV-mXBP-1S, respectively (data not shown).

To ensure that the proteins expressed played their physiological roles of regulating a subset of UPR genes, the above plasmids were co-transfected with BiP-Luc, a reporter plasmid encoding firefly luciferase under the control of the human BiP promoter. It has been shown that XBP-1S binds to the ER stress element within the BiP promoter and activates BiP transcription, whereas XBP-1U has no effects on BiP expression (Yoshida et al., 1998). Human ATF6α(1-373), a known transcription activator for BiP containing the N-terminal domain of the protein ATF6α (a.a. 1-373), was used as a positive control (Yoshida et al., 2000). The data show that ATF6α(1-373) induced a 20-fold increase in luciferase expression in CHO-K1 cells (Fig. 1). In comparison, XBP-1S caused a 15-fold increase in luciferase activity while XBP-1U
showed no activation on the reporter gene (Fig. 1). Furthermore, we evaluated the expression levels of ERdj4, an ER chaperone as well as an XBP-1-dependent UPR target gene (Lee et al., 2003), in CHO cells overexpressing GFP, XBP-1U, and XBP-1S (Table 1). Results from qRT-PCR indicate that cells transfected with CMV-mXBP-1S plasmid show a two-fold increase in ERdj4 mRNA level, relative to cells transfected to CMV-GFP and CMV-mXBP-1U plasmids (Table 1). Taken together, the results from the BiP-Luc luciferase assay and qRT-PCR analysis of Erdj4 confirm the biological functionality of the cloned XBP-1U and XBP-1S in CHO cells. In particular, the cloned XBP-1S was shown to be able to upregulate the secretory machinery of the cell, as evidenced by activating the BiP promoter and increasing the endogenous ERdj4 expression.

The antibody-producing stable cell line, CHO-MAb, was then transiently transfected with CMV-mXBP-1S to investigate the effects of XBP-1S overexpression on MAb production. Culture supernatant was collected daily up to three days post-transfection and analyzed by ELISA. No improvement on MAb titer was detected when XBP-1S was overexpressed (Fig. 2A). A similar set of experiments was carried out in CHO-IFNγ stable cells. Results show that XBP-1S overexpression had no significant effects on IFNγ production in this cell line (Fig. 2B). The effects of XBP-1S overexpression was then further investigated by titrating the amount of CMV-mXBP-1S transfected in CHO-EPO and CHO-IFNγ stable cell lines. Similarly, overexpression of XBP-1S (at different expression levels) caused little or no detectable improvement in EPO or IFNγ (Fig. 3A and B). Contrary to our expectation, overexpression of XBP-1S has no detectable effects on protein productivity in CHO stable cell lines.
Overexpression of XBP-1S significantly enhanced transient EPO production in EPO-saturated CHO-K1 cells

As it is possible that the productivity of the stable cell lines used above may not be limited by protein secretion, it became necessary then to test the effects of XBP-1S overexpression over a wider range of protein expression level. This productivity range was achieved by transiently transfecting CHO-K1 cells with varying amounts of CMV-EPO plasmid. Two days post-transfection, EPO titer of up to 33.7 IU/ml was achieved in transient production cultures (Fig. 4), much higher than the titer of 0.7 IU/ml achieved in a two-day culture of the CHO-EPO stable cell line (Fig. 3B). In addition, it was observed that EPO titer increased in a dosage-dependent manner at low plasmid dosages, reaching a plateau when more than 0.2 μg of CMV-EPO plasmids was used (Fig. 4). The corresponding expression levels of EPO in transfected CHO-K1 cells were monitored by qRT-PCR. The mRNA levels of EPO were shown to be well-correlated with the amount of CMV-EPO transfected, with no sign of leveling off (data not shown). These observations suggest that, at high plasmid dosages, the secretory pathway may have been saturated and secretion may have become the rate-limiting step for protein production. At this condition, XBP-1S overexpression could enhance EPO secretion significantly.

To test this hypothesis, CHO-K1 cells were co-transfected with varying amounts of CMV-mXBP-1S or CMV-mXBP-1U and 0.2 μg of CMV-EPO, the plasmid dosage at which the saturated production of EPO was achieved (Fig. 4). As anticipated, overexpression of XBP-1S increased EPO titers by up to 2.5-fold, while XBP-1U showed no improvement on production (Fig. 5A, transient). In contrast, no such increase was observed in the CHO-EPO stable cell line with low EPO productivity (Fig. 5A,
To further characterize the enhancing effect of XBP-1S in transient production, CHO-K1 cells were transfected with 0.05 µg of CMV-mXBP-1S and varying amounts of CMV-EPO (Fig. 5B). As shown in Fig. 5B, overexpression of XBP-1S caused significant improvement in EPO titers (up to 4-fold) compared to the other control groups (blank vector, XBP-1U, and GFP). Most importantly, the enhancing effect of XBP-1S was only observed in the EPO-saturated CHO-K1 cells (Fig. 5B, 0.2-0.4 µg CMV-EPO). In contrast, no such improvement was observed when XBP-1S was overexpressed in non-saturated cells (see Fig. 5B, 0.1 µg CMV-EPO). These results confirm our hypothesis that XBP-1S can further improve protein production only when the accumulated amount of the newly synthesized recombinant protein exceeds the secretory capacity of host cells.

Since XBP-1S is a transcription activator, it is possible that XBP-1S may improve EPO production at the transcriptional level by activating the CMV MIE promoter that drives EPO expression. To determine the effects of XBP-1S overexpression on the level of EPO transcripts, CHO-K1 cells were transiently transfected with 0.1 µg CMV-mXBP-1S and various dosages of CMV-EPO. The levels of EPO mRNA were quantitated using qRT-PCR. Results show that overexpression of XBP-1S had no significant effects on EPO transcripts regardless of the amount of CMV-EPO plasmid used (Table 2). To examine the effects of XBP-1 overexpression on protein synthesis, an identical set of experiments was carried out using CMV-Luc plasmid, a luciferase reporter plasmid in which the expression of luciferase is under the control of the CMV MIE promoter. Similarly, no increase in luciferase expression was detected when cells were co-transfected with XBP-1S plasmids (data not shown). Collectively, these results suggest...
that the titer improvement observed is a result of XBP-1S modulating the cellular secretory pathway.

**Overexpression of XBP-1S significantly enhanced transient EPO production in EPO-saturated NS0 myeloma cells**

The effects of XBP-1S overexpression were further examined in other cell lines. Given the role of XBP-1S in plasma cell differentiation, we expected that XBP-1S might exhibit similar enhancement in recombinant protein production in cells of B-cell lineage. This strategy was thus tested on NS0 myeloma cells. Similar to the approach taken in CHO-K1 cells, NS0 cells were transiently transfected with varying amounts of CMV-EPO plasmid. The number of surviving cells and culture recovery post-electroporation varied between different transfection reactions (Fig. 6A). To minimize the effects of these variations, cell number of each culture was determined daily and EPO titer was normalized to the corresponding integrated viable cell density (Fig. 6A). Within the range of plasmid dosage used, a dosage-dependent increase of specific EPO productivity of up to 4.1 IU/10⁹ cells/day (approximately 28 pg/cell/day) was observed (Sun et al., 2006) (Fig. 6B). However, the increase in specific productivity between 2.0 and 3.0 μg of CMV-EPO plasmid dosage seemed to be slower compared to that for smaller dosages (Fig. 6B, between 1.0 and 2.0 μg), suggesting that saturation may be reached with 3.0 μg or more of CMV-EPO plasmid. As a plasmid dosage of more than 3.0 μg posed severe cytotoxicity (data not shown), total plasmid amount was kept to 3.0 μg in subsequent experiments. NS0 cells were co-transfected with 2.8 μg of CMV-EPO and up to 0.2 μg of CMV-mXBP-1S plasmids. Results show that specific EPO productivity increased from
4.0 IU/10^6 cells/day (approximately 28 pg/cell/day) to 7.8 IU/10^6 cells/day (approximately 56 pg/cell/day) when XBP-1S was overexpressed (Fig. 6 C and D). The observations made in NS0 cells further confirmed those in CHO-K1 cells, supporting our hypothesis that overexpression of XBP-1S can significantly improve recombinant protein production when high expression levels of the proteins exceed the secretory capacity of the host cell.
DISCUSSION

As most biopharmaceuticals are secreted recombinant products, cellular engineering strategies involving modulation of the secretory capacity could be useful for improving recombinant protein production. In this study, the effects of overexpression of mouse XBP-1S, a global regulator of the secretory pathways, on the production of recombinant biopharmaceuticals, including MAb, IFNγ, or EPO, in CHO and NS0 cell lines were examined. Contrary to previous report (Tigges et al., 2006), the effects of XBP-1S overexpression on protein productivity was found to depend on the expression levels of recombinant proteins. Specifically, our data clearly demonstrate two distinct production regimes – a saturating regime where the level of secreted EPO could be increased by overexpression of XBP-1S (Fig. 5 and 6), and a non-saturating regime where the same strategy produced no detectable effects on protein titers (Fig. 2 and 3). In the non-saturating regime, protein production may mainly be regulated at transcriptional or translational stages. Under this condition, a strategy aimed at up-regulating the secretory capacity would understandably not impact the overall recombinant protein production. In contrast, in the saturating regime, the yield of secreted protein is limited by a secretion bottleneck, as evidenced by protein titer not increasing with increased recombinant protein expression (Fig. 4). Higher protein titers would be expected when the secretory pathway of saturated cells is modulated by overexpressing XBP-1S. Based on this study, we propose a qualitative model for the effects of XBP-1S overexpression on recombinant protein production (Fig. 7).

Compared to EPO titers in the transient transfection assays (up to 33.7 IU/ml, Fig. 4), the titer achieved by the CHO-EPO stable cell line (0.7 IU/ml) falls within the non-
saturating regime of EPO production (Fig. 3). This helps to explain why overexpression of XBP-1S in CHO-EPO cells could not increase EPO titer significantly. Furthermore, unpublished in-house data suggest that both the CHO-IFNγ and CHO-MAb cells used in this study may be regulated by transcription, but not limited by secretion. These observations account for the lack of positive effects of XBP-1S in these cells.

Our results are in contrast to those obtained by Tigges et al. (Tigges et al., 2006). In the stable SEAP-/XBP-1S-producing cell lines, the authors detected 3- to 5-fold increases in SEAP production when compared to the parental SEAP-producing cells. However, the SEAP titers in the cell lines were very low (comparable to the non-SEAP producing CHO-K1 cells), suggesting that the production of SEAP might mainly be regulated at transcriptional level, but not at secretion stage (Tigges et al., 2006). To address the correlation between expression levels of recombinant proteins and effects of XBP-1S in detail, the authors performed a separate experiment involving SEAP-producing CHO stable cell lines, in which SEAP titers could be modulated using a tetracycline-dependent expression system. XBP-1S was found to consistently improve SEAP production under all different expression levels of SEAP. Based on the results, the authors concluded that the increase in recombinant protein production caused by XBP-1S overexpression was independent of expression levels of the proteins (Tigges et al., 2006). Their conclusion stands in stark contrast to our findings, which clearly demonstrate that the expression level of recombinant proteins is indeed a key determinant in the effects of XBP-1S. Based on this premise, Tigges’ observations seem counterintuitive as the production of SEAP in this case was clearly transcriptionally regulated, but not limited by
secretion. It remains to be shown how targeting XBP-1S could have benefited a transcription-regulated protein production regime.

In general, the differences observed in Tigges’ and our current study may be attributable to several reasons. First of all, XBP-1 proteins from different species were used in the two studies. Human XBP-1 was used in the former study whilst the mouse homolog was used in our experiments. Although highly unlikely, the minor sequence variation between the two homologs may have contributed to the difference in observations. Secondly, the effects of XBP-1S on productivity may be specific to particular recombinant proteins. As protein production is a complicated process regulated by several biological pathways including transcription, translation, post-translational modification, protein folding and assembly, and secretion, the production of different proteins may impose different metabolic and secretory burdens on the cells. As such, XBP-1S overexpression may exert different effects on the production of different recombinant proteins. Indeed, the protein-specific effects of XBP-1S overexpression have been reported. Overexpression of Hac1p, the yeast homolog of XBP-1, was shown to increase the secretion of invertase and Bacillus amyloliquefaciens α-amylase in yeast but had no effects on that of Trichoderma reesei endoglucanase (Valkonen et al., 2003).

In conclusion, when compared to genetic engineering strategies targeting single ER components like BiP and PDI, modulation of the UPR through XBP-1S overexpression may be more effective owing to the role of XBP-1S in regulating multiple critical genes in the secretory pathway in a concerted fashion. Microarray analyses of B cells with ectopic expression and knock-down of XBP-1S identified XBP-1S target genes such as heat shock protein 40 homolog, signal recognition peptide, sec61a homolog,
vesicle docking protein, mannosidase acetylglucosaminyltransferase 2, ER-associated
degradation mannosidase-like protein, and many other proteins that are involved in
folding, translocation, trafficking, glycosylation, and degradation (Shaffer et al., 2004).
The outworking of this global regulation is demonstrated in the development of
physiological professional secretors such as the plasma cell (Iwakoshi et al., 2003b;
Iwakoshi et al., 2003a; Reimold et al., 2001; Shaffer et al., 2004). Our study shows that
this physiological regulatory mechanism can in fact be applied in host cell engineering
strategies aimed at increasing recombinant protein production. XBP-1S overexpression is
not a strategy that indiscriminately enhances recombinant protein production in all
instances; it is nonetheless one which is effective in relieving secretory bottlenecks that
may be encountered in some production systems.
ACKNOWLEDGEMENTS

We would like to thank Dr. Kazutoshi Mori for his kind provision of the Bip-Luc plasmid, Dr. Janet Chusainow, Dr. Yang Yuan Sheng, Poh Choo Toh, Jessna Yeo, Sing Fee Lim, and Maybelline Giam for cell line creation and other forms of technical inputs.
REFERENCES


Shaffer AL, Shapiro-Shelef M, Iwakoshi NN, Lee AH, Qian SB, Zhao H, Yu X, Yang L, Tan BK, Rosenwald A, Hurt EM, Petroulakis E, Sonenberg N, Yewdell JW,


FIGURE LEGENDS

Figure 1
Transcriptional activity of cloned XBP-1S and XBP-1U in CHO-K1 cells. 0.05 μg of BiP-Luc, a firefly luciferase reporter plasmid under the control of human BiP promoter, was co-transfected with 0.1 μg of an indicated plasmid [i.e. blank, ATF6α (1-373), mXBP-1S or mXBP-1U vectors]. Human ATF6α (1-373), a known transcriptional activator of BiP, containing the N-terminal domain of ATF6α (a.a. 1-373) was used as a positive control, while the blank vector as a negative control. Luciferase activity was assayed 2 days post-transfection. Error bars indicate standard deviations.

Figure 2
Effects of XBP-1S overexpression on recombinant protein production of producing cell lines. CHO cells stably expressing (A) a monoclonal antibody and (B) human IFNγ were each transiently transfected with 5 μg of blank vector or CMV-mXBP-1S expression plasmid by electroporation and FuGENE 6 transfection reagent respectively. Both experiments were carried out in 6-well format. Culture supernatant was collected everyday up to three days and two days post-transfection, respectively. Protein titer was quantitated by ELISA. Error bars indicate standard deviations.

Figure 3
Titration of CMV-mXBP-1S in producing cell lines. CHO cells stably expressing (A) human IFNγ and (B) human EPO were transiently titrated with indicated amounts of CMV-mXBP-1S expression plasmid. Culture supernatant was collected 2 days post-
transfection and protein titer was quantitated by ELISA. Error bars indicate standard deviations.

Figure 4
Transient production of EPO in CHO-K1 cells. CHO-K1 cells were transiently transfected with the indicated amounts of CMV-EPO. Culture supernatant was collected 2 days post-transfection and protein titer was quantitated by ELISA. Error bars indicate standard deviations.

Figure 5
Effects of XBP-1S overexpression on transient production of EPO in CHO cells. (A) CHO-K1 cells transfected with indicated amounts of CMV-mXBp-1S and CMV-mXBp-1U with 0.2 μg of CMV-EPO plasmids. For comparison, CHO-EPO cells were transfected with the same amounts of CMV-mXBp-1S and CMV-mXBp-1U plasmids. Culture supernatant was collected 2 days post-transfection and analyzed by ELISA. Error bars indicate standard deviations. (B) CHO-K1 cells were transiently transfected with a fixed amount of a blank plasmid, CMV-mXBp-1S, CMV-XBP-1U, or CMV-GFP together with indicated amounts of CMV-EPO. Blank vector, XBP-1U, and GFP were used as negative controls. Culture supernatant was collected 2 days post-transfection and protein titer was quantitated by Western blotting. Band volume was quantitated using a densitometer (Model: GS-800; Bio-Rad).

Figure 6
Effects of XBP-1S overexpression on transient production of EPO in NS0 cells. (A, B) Transient titration of NS0 cells with indicated amounts of CMV-EPO plasmid. (C, D) Transient co-transfection of NS0 cells with 2.8 µg of CMV-EPO and indicated amounts of CMV-mXBP-1S plasmids. The total amount of transfected plasmid was kept constant (i.e. 3 µg) by adjusting with the blank vector. Viable cell numbers were counted daily (A, C). Day 0 viable cell count was obtained 2-3 hours post-electroporation, the variation in cell number indicates difference in cell survival rate after electroporation. Culture supernatant was collected 2 days post-transfection and analyzed by ELISA. Specific EPO productivity was determined by normalizing calculated EPO titer with integrated viable cell densities of transfected cultures. Error bars indicate standard errors propagated from standard deviations of ELISA measurements.

Figure 7
Qualitative model of the effects of overexpression of XBP-1S on recombinant protein production.
Table 1: ERdj4 expression in CHO-K1 cells overexpressing GFP, XBP-1U, and XBP-1S.
CHO-K1 cells in T25 flasks were transfected with 10 µg of the respective expression plasmids. Total RNAs were isolated 2 days post-transfection for qRT-PCR analysis.

<table>
<thead>
<tr>
<th>Transfected Plasmid</th>
<th>ΔC_T</th>
<th>Fold Change Average # (range) Ⅱ</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMV-GFP</td>
<td>6.45 ± 0.14</td>
<td>1.00 (0.88 – 1.14)</td>
</tr>
<tr>
<td>CMV-mXBP-1U</td>
<td>6.70 ± 0.13</td>
<td>0.84 (0.74 – 0.96)</td>
</tr>
<tr>
<td>CMV-mXBP-1S</td>
<td>5.43 ± 0.08</td>
<td>2.02 (1.81 – 2.25)</td>
</tr>
</tbody>
</table>

* Results shown have been normalized by subtracting the C_T values of ERdj4 to that of actin.

# Fold change relative to ERdj4 transcript level in CMV-GFP transfected cells. Average fold change was calculated using the equation:

\[
\text{Fold Change} = 2^{-\Delta\Delta C_T}
\]

Ⅱ Range of fold change was calculated by incorporating the standard errors of \(\Delta\Delta C_T\) values:

\[
\text{Fold Change (lower limit)} = 2^{-\left(\Delta\Delta C_T + \text{Standard Error}\right)}
\]

\[
\text{Fold Change (upper limit)} = 2^{-\left(\Delta\Delta C_T - \text{Standard Error}\right)}
\]
Table 2: Effect of XBP-1S overexpression on transcription of EPO. CHO-K1 cells in 96-well plate were transfected with indicated amounts of CMV-EPO plasmids. Total RNAs from 4 replicates were isolated and pooled for qRT-PCR analysis 2 days post-transfection.

<table>
<thead>
<tr>
<th>CMV-EPO (µg)</th>
<th>ΔCₜ *</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.05</td>
</tr>
<tr>
<td>Blank Vector</td>
<td>1.73 ± 0.24</td>
</tr>
<tr>
<td>CMV-mXBP-1S</td>
<td>2.45 ± 0.18</td>
</tr>
</tbody>
</table>

*: Results shown have been normalized by subtracting the Cₜ values of EPO to that of actin.
![Bar graph showing MAb (µg/ml) levels for Blank Vector and CMV-mXB1P-1S over 3 days post-transfection.](image)

- **Days Post-transfection**: 1, 2, 3
- **Y-axis**: MAb (µg/ml) ranging from 0 to 45
- **Legend**:
  - Blank Vector
  - CMV-mXB1P-1S

**Note**: The graph shows a significant increase in MAb levels for both groups from Day 1 to Day 3, with Blank Vector showing a smaller increase compared to CMV-mXB1P-1S.
<table>
<thead>
<tr>
<th>CMV-EPO (µg)</th>
<th>0.1</th>
<th>0.2</th>
<th>0.3</th>
<th>0.4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank Vector</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
<td><img src="image4.png" alt="Image" /></td>
</tr>
<tr>
<td>CMV-GFP (fixed at 0.05 µg)</td>
<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
<td><img src="image7.png" alt="Image" /></td>
<td><img src="image8.png" alt="Image" /></td>
</tr>
<tr>
<td>CMV-mXBP-1U (fixed at 0.05 µg)</td>
<td><img src="image9.png" alt="Image" /></td>
<td><img src="image10.png" alt="Image" /></td>
<td><img src="image11.png" alt="Image" /></td>
<td><img src="image12.png" alt="Image" /></td>
</tr>
<tr>
<td>CMV-mXBP-1S (fixed at 0.05 µg)</td>
<td><img src="image13.png" alt="Image" /></td>
<td><img src="image14.png" alt="Image" /></td>
<td><img src="image15.png" alt="Image" /></td>
<td><img src="image16.png" alt="Image" /></td>
</tr>
</tbody>
</table>

![Bar Chart](chart.png)

- **Blank Vector**
- **CMV-GFP**
- **CMV-mXBP-1U**
- **CMV-mXBP-1S**

Relative Band Volume

<table>
<thead>
<tr>
<th>CMV-EPO (µg)</th>
<th>0.1</th>
<th>0.2</th>
<th>0.3</th>
<th>0.4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank Vector</td>
<td><img src="image17.png" alt="Image" /></td>
<td><img src="image18.png" alt="Image" /></td>
<td><img src="image19.png" alt="Image" /></td>
<td><img src="image20.png" alt="Image" /></td>
</tr>
<tr>
<td>CMV-GFP</td>
<td><img src="image21.png" alt="Image" /></td>
<td><img src="image22.png" alt="Image" /></td>
<td><img src="image23.png" alt="Image" /></td>
<td><img src="image24.png" alt="Image" /></td>
</tr>
<tr>
<td>CMV-mXBP-1U</td>
<td><img src="image25.png" alt="Image" /></td>
<td><img src="image26.png" alt="Image" /></td>
<td><img src="image27.png" alt="Image" /></td>
<td><img src="image28.png" alt="Image" /></td>
</tr>
<tr>
<td>CMV-mXBP-1S</td>
<td><img src="image29.png" alt="Image" /></td>
<td><img src="image30.png" alt="Image" /></td>
<td><img src="image31.png" alt="Image" /></td>
<td><img src="image32.png" alt="Image" /></td>
</tr>
</tbody>
</table>