Enhanced IgG1 production by overexpression of nuclear factor kappa B inhibitor zeta (NFKBIZ) in Chinese hamster ovary cells

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Abstract Several engineering strategies have been employed to improve the production of therapeutic recombinant proteins in Chinese hamster ovary (CHO) cell lines. We have focused on unfolded protein response-based engineering and reported that ATF4 overexpression increases protein production. In this study, transcriptome analysis of ATF4-overexpressed CHO cells was performed using high-coverage expression profiling, to search for another key factor contributing to recombinant protein production. We observed the upregulated expression of transcription factor, nuclear factor (NF)-kappa-B inhibitor zeta (NFKBIZ or Ikb ζ), in ATF4-overexpressed cells. A total of 1917 bp of CHO NFKBIZ cDNA was cloned,

and two stable cell lines overexpressing NFKBIZ were constructed. We investigated the effects of NFKBIZ on IgG1 production in CHO cells. Although the two stable cell lines, NFKBIZ-A and -B, had the opposite phenotypes in cell growth, the specific IgG1 production rate of both cell lines was enhanced by 1.2–1.4-fold. In the NFKBIZ-A cell line, the synergistic effect between enhanced viable cell density and improved specific IgG1 production rate brought about a large increase in the final IgG1 titer. Luciferase-based NF- κ B signaling assay results suggest that altered p50/p50 signaling seems to be due to the opposite phenotypes in cell growth. No difference was observed in the translational levels and intracellular assembly states of IgG1 between mock and two NFKBIZ cell lines, indicating that the secretion machinery of correctly folded IgG1 was enhanced in NFKBIZ-overexpressing cell lines.

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Introduction

Chinese hamster ovary (CHO) cells are the most representative cell line for the industrial production of monoclonal antibodies because they can produce

correctly folded proteins with post-translational modifications (Wurm 2004; Omasa et al. 2010; Li et al. 2010). Despite the recent progress in high-yielding recombinant antibody of 10 g/L, it remains difficult to construct highly productive CHO cell lines. Genomic DNA elements were introduced into an expression plasmid to enhance stable gene expression: for example, insulators, expression augmenting sequence element, scaffold/matrix attachment region, ubiquitous chromatin opening elements, stabilizing and anti-repressor elements (Cacciatore et al. 2010). Recently a novel regulatory motif from the CHO DR1000L-4N cell line was identified (Takagi et al. 2017). Cell engineering approaches also have been attempted to improve antibody productivity (Fischer et al. 2015). Cell engineering approaches comprise the improvement of apoptosis, metabolic process, glycosylation, cell cycle regulation, protein folding and secretion. Among these, unfolded protein response (UPR)-based engineering has been the one of the main targets of engineering research because of the structural complexity of antibodies (Khan and Schroder 2008; Hussain et al. 2014). The UPR signal is transduced by three sensor proteins, IRE1, ATF6 and PERK, in response to the accumulation of unfolded or misfolded proteins in the endoplasmic reticulum (ER), resulting in the induction of chaperone expression, translational attenuation, protein degradation, autophagy and apoptosis to maintain cell homeostasis. Overexpression of X-box binding protein (Xbp1) involved in the IRE1 pathway has been reported to increase the product titer (Tigges and Fussenegger 2006; Becker et al. 2008). In transient expression of a difficult-to-express antibody, BiP, ATF6c and XBP1s co-expression increased specific productivity and reduced cell growth (Pybus et al. 2014). We have focused on the PERK pathway in UPR (Ohya et al. 2008; Omasa et al. 2008; Haredy et al. 2013). Previously we reported the increased specific productivity in recombinant CHO cell lines by overexpression of activating transcription factor 4 (ATF4; Ohya et al. 2008; Haredy et al. 2013). In UPR, the protein translation step is attenuated by phosphorylation of eIF2 α to avoid an excess accumulation of polypeptide in the ER. ATF4 expression induces the eIF2 α dephosphorylation by activating GADD34 in recombinant CHO cells (Ohya et al. 2008; Omasa et al. 2008), which is thought to account for the increased specific productivity. Aside from the intracellular mechanisms, little is known about the genome-wide

transcriptional analysis of UPR-engineered CHO cells producing recombinant proteins; it remains unclear whether there are unidentified mechanisms or genes contributing protein production in UPR engineering. An omics approach has recently been used for characterizing CHO cells for host cell engineering (Datta et al. 2013; Kildegaard et al. 2013). In the present study, transcriptome analysis was performed using high-coverage expression profiling (HiCEP). The HiCEP method was originally developed by the Transcriptome Profiling Group of the National Institute of Radiological Sciences (Fukumura et al. 2003), and it has advantages over microarray or DNA chip analysis in its high sensitivity, reproducibility and high coverage of transcripts containing known-gene, unknown-gene and non-coding regions. However, a target sequence of an up- or downregulated transcript must be cloned after analysis. Here we report the upregulated expression of the transcription factor nuclear factor (NF)-kappa B inhibitor zeta (NFKBIZ) in ATF4-overexpressing CHO cells. Cloned DNA encoding CHO NFKBIZ was stably introduced into the CHO cell line producing IgG1. NFKBIZ-overexpressing cell lines showed a significant increase in IgG1 specific productivity. Although the relationship between the increased specific productivity and NF-kappa B pathway should be further investigated, NFKBIZ-mediated translational and secretory control is considered to contribute to the enhanced IgG1 production.

Materials and methods

High-coverage expression profiling (HiCEP)

Total RNA for HiCEP analysis was extracted from control 13D-35D mock and 13D-35D ATF4-G cell lines (Ohya et al. 2008). The cell lines were cultivated in α -MEM medium supplemented with 0.5% FBS (JRH Biosciences, Lenexa, KS, USA), 1 μ M MTX (Sigma-Aldrich, St. Louis, MO, USA) and 250 μ g/mL G418 (Sigma-Aldrich). Total RNA was prepared with the RNeasy MiniKit (Qiagen, Hilden, Germany) using DNase I (Takara Bio, Shiga, Japan) treatment. HiCEP was carried out at the National Institute of Radiological Science (NIRS; Chiba, Japan). The basic protocol was developed by the HiCEP unit team at NIRS (Fukumura et al. 2003), and the following procedure

was performed by the team's technical services. Briefly, cDNA was synthesized from the total RNA by reverse transcriptase with biotinylated oligo(dT) primer. First-strand cDNA was synthesized into double-stranded cDNA with RNaseH, DNA polymerase I and DNA ligase. Double-stranded cDNA was digested with *Msp* I, and the digested cDNA was purified with avidin-conjugated magnetic beads. The 5'-end of products was capped with the synthetic *Msp* I adapter, followed by *Mse* I digestion. The digested product was released from magnetic beads and capped with the *Mse* I adapter. After preparation of the template cDNA, selective PCR was performed with *Mse* I and fluorescence-labeled *Msp* I adapter primers. The PCR products were injected into the ABI PRISM 3100 electrophoresis system (Applied Biosystems, Foster City, CA), USA. Duplicate HiCEP analysis for RNAs from mock and ATF4-overexpressing cells was carried out. The expression level of the corresponding gene transcript was represented by the fluorescence intensity of each peak. For peak normalization between electrophoresis, the global normalization program developed by Maze Inc. (Tokyo, Japan) was used.

cDNA cloning of CHO NF-kappa B inhibitor zeta

Several upregulated transcripts observed in HiCEP analysis were fractionated and analyzed by direct-sequencing (Technical services of Messenger Scape, Tokyo, Japan). Based on the obtained 260 bp of sequence No. 3, a complete cDNA sequence was prepared with 3'-Full Race Core Set and 5'-Full RACE Core Set (Takara Bio) according to the manufacturer's protocol. Total RNA from 13D-35D ATF4-G was used as a template for reverse transcription. Following first and nested PCR with PrimeSTAR Max and LA Taq DNA polymerase (Takara Bio), PCR products were subcloned into pBluescript (Agilent Technologies, Santa Clara, CA, USA) or pMD20 (Takara Bio), followed by DNA sequencing. Segment sequences from 3'- and 5'-RACE were reassembled into full-length cDNA of the upregulated 260 bp transcript, resulting in the 1.9 kbp of NF-kappa B inhibitor zeta (NFKBIZ). The cDNA of CHO NFKBIZ was cloned from the cDNA pool prepared from mRNA of the 13D-35D cell subjected to 5 µg/mL tunicamycin treatment (Wako Pure Chemicals, Osaka, Japan) using the following primer sets: 5'-CGAGGTTGAGCCCCACATG-3' and

5'-CTAGTACGGTGGTGCTCGCT-3'. The 1.9 kbp PCR product was cloned into pBluescript, and its sequence was consistent with that of full-length cDNA from 3'- and 5'-RACE. The CHO NFKBIZ cDNA was subcloned into the *Hind* III and *Xba* I site of the pcDNA 3.1/Hygro(+) vector (Thermo Fisher Scientific, Waltham, MA, USA).

Construction of NFKBIZ-overexpressing CHO cells

The pcDNA3.1-NFKBIZ/Hygro(+) vector was transfected into CHO-HcD6 (Onitsuka and Omasa 2015) with X-tremeGENE9 DNA transfection reagent (Roche, Basel, Switzerland). A mock cell line was constructed by transfection of pcDNA3.1/Hygro(+). Transfected cells were cultivated in RDF medium with 10% FBS, 15 µg/mL puromycin (Invivogen, San Diego, CA, USA) and 500 µg/mL hygromycin (Wako Pure Chemicals). Single clones with hygromycin resistance were isolated with a penicillin cup. Genomic DNA and total RNA from single clones were prepared with the High Pure PCR Template Preparation Kit and High Pure RNA Isolation Kit (Roche), respectively, and cDNA was prepared with the PrimeScript First-strand cDNA Synthesis Kit (Takara Bio). Genomic integration of pcDNA3.1-NFKBIZ/Hygro(+) was confirmed by PCR with Emerald Amp (Takara Bio) and the primer sets designed from pcDNA 3.1/Hygro(+). Transcription of CHO NFKBIZ was confirmed by RT-PCR with Emerald Amp (Takara Bio) and the following primer sets: 5'-TTGGCCGGCAGCAAAGAGGAC-3' and 5'-CTAGTACGGTGGTGCTCGCTG-3', which were designed from the cloned DNA sequence. CHO NFKBIZ-overexpressing clones and mock cells were adapted to the serum-free medium TOP2 (Irvine Scientific, Santa Ana, CA, USA) supplemented with 8 mM L-glutamine, 15 µg/mL puromycin and 250 µg/mL hygromycin.

Cell culture and antibody analysis

The cell lines were cultured in 500-mL Erlenmeyer flasks (Corning Inc., Corning, NY, USA) with a working volume of 100-mL serum-free medium TOP2. Three independent cultures for one cell line were performed. The flasks were incubated at 37 °C and 80% humidity, with shaking at 80 rpm in a Climo-shaker ISF1-X (Kuhner, Basel, Switzerland). Viable cell density and cell viability were measured using Vi-

CELL XR (Beckman Coulter, Fullerton, CA, USA). Antibody production was measured using biolayer interferometry with an Octet QK system and anti-human IgG Fc sensor (Fortebio, MenloPark, CA, USA). IgG1, purified with the HiTrap rProteinA FF column (GE Healthcare, Little Chalfont, UK) from the culture supernatant, was used as reference standard. The specific growth rate (μ) and the specific antibody production rate (p) were calculated as described elsewhere (Omasa et al. 1992). Oligomeric states of secreted IgG1 were analyzed by size exclusion chromatography (SEC) with the Superdex 200 column (GE Healthcare). The relative content of the IgG1 monomer was estimated by peak deconvolution analysis using Igor software (Wavemetrics Inc., Lake Oswego, OR, USA). IgG1 polypeptides in CHO cells were analyzed by capillary electrophoresis immunoassay using the Wes and Wes-Rabbit 12–230 kDa Master Kit (ProteinSimple, San Jose, CA, USA). Cell lysate was prepared with M-PER Mammalian Protein Extraction Reagent (ThermoFisher Scientific). Human IgG-Fc Fragment Antibody (A80-105A, Bethyl Laboratories, Montgomery, TX, USA) and Human Kappa Light Chain Antibody (A80-105A, Bethyl Laboratories) were used as the primary antibodies for detection.

Quantitative real-time PCR

Transcriptional levels of CHO NFKBIZ and UPR-related genes were measured using Step ONE Plus Real-time PCR system (Life Technologies). Total RNA was extracted with High Pure RNA Isolation Kit (Roche), and cDNA was prepared with the PrimeScript First-strand cDNA Synthesis Kit (Takara Bio). PCR reaction was performed using the SYBR Green PCR Master MIX (Applied Biosystems) and corresponding primer sets (Table 1). β -Actin was used to normalize the quantification of mRNA expression.

Reporter assay for NF- κ B activity measurement

Activation of the NF- κ B pathway was investigated based on the luciferase reporter assay. The SV40 promoter sequence was amplified by PCR using corresponding primers with *Pst* I site and pSV2-dhfr (ATCC 37146) as the template, and the PCR products were inserted into the *Pst* I site of pELuc-test promoter-less vector (Toyobo, Osaka Japan). Synthetic oligonucleotides including six-fold repeats of

Table 1 Primers used for real-time PCR

Name	Sequence (5' → 3')
β -Actin forward	ACCATGGATGATGATATCGC
β -Actin reverse	ACATGGCTGGGGTGTGAAG
NFKBIZ forward	TCCTCCAGCATCCCACAAC
NFKBIZ reverse	AGTGACAGGGAAGTACCCAAAG
ATF4 forward	TTCTCCAGCGACAAGGCTAAG
ATF4 reverse	GCACTGACCAACCCATCCA
CHOP forward	GGGAGCTGGAAGCCTGGTAT
CHOP reverse	GGGACCCCCATTTTCATCTG
GADD34 forward	CCTGGTCTGCAAAGTGCTGAT
GADD34 reverse	CCAGCTCAGTCACTCCCTCTTC
GRP78 forward	CCTGCGTTGGTGTGTTCAG
GRP78 reverse	GCGGTTGCCCTGATCGT
IgG1 HC forward	CCCCGAGGTGAAGTTCAACTG
IgG1 HC reverse	GCGGTACGTCGAGTTGTACTG
IgG1 LC forward	GGAGGACTTCGCCACCTACT
IgG1 LC reverse	ACGGTCCGCTTGATCTCCA

NF-kappa B binding sequence (GGGAAGTTCC)₆ for p65/p50 and (GGGAAAATCC)₆ for p50/p50 were ligated into *Xho* I and *Bgl* II sites of the pEluc-SV40 vector. The resulting reporter assay vectors were designated as pEluc-(p65/p50)-SV40 and pEluc-(p50/p50)-SV40. The assay vector and pSV- β -Galactosidase Control Vector (Promega, Madison, WI, USA) were co-transfected into mock and NFKBIZ-overexpressing cells using PEI max (Polysciences, Warrington, PA, USA). After 24 h of transfection, the luciferase assay was performed with Emerald Luc Luciferase Assay Reagent (Toyobo), and the transfection efficiency was evaluated using the β -Galactosidase Enzyme Assay System (Promega). All measurements were conducted with EnSpire multi-label reader (PerkinElmer, Waltham, MA, USA).

Results and discussion

Upregulated expression of NF-kappa B inhibitor zeta in ATF4-overexpressing cells

An overview of the study method is given in Fig. 1. The first step involves transcriptome analysis of the UPR-engineered cell by HiCEP (Fukumura et al. 2003). We investigated differential expression in

Fig. 1 Schematic diagram of the study design. Upregulation of NF-kappa B inhibitor zeta (NFKBIZ or IκBζ) was observed in the ATF4-overexpressing CHO cell line using HiCEP analysis.

ATF4-overexpressing CHO 13D-35D (Ohya et al. 2008). ATF4 is a key regulator of the PERK pathway in UPR, and we have reported the enhanced production of recombinant proteins by ATF4 overexpression (Ohya et al. 2008; Haredy et al. 2013). Reverse-transcribed cDNA was subjected to amplified fragment-length polymorphism PCR. PCR products were analyzed by capillary electrophoresis, where one gene transcript corresponds to one peak in profiling. The results of the HiCEP analysis are summarized in the supplementary file. We observed seven upregulated transcripts showing an increased peak area. Among these, three transcript sequence peaks were successfully fractionated and cloned. We concluded, based on several lines of evidence, that transcripts 1 and 2 were unrelated to ATF4 overexpression. A 90-bp segment of transcript 1 (supplementary file) was not upregulated by tunicamycin treatment in CHO 13D-35D (data not shown), indicating that the transcript is independent of UPR signaling. The 5'-end sequence of

Cloned CHO Nfkbiz was overexpressed in the recombinant CHO cell line producing IgG1, and the effects were investigated

transcript 2 (81 bp, supplementary file) was obtained by 5'-RACE methods and was a part of the transcription sequence of the ATF4 overexpression plasmid derived from CHO 13D-35D ATF4 cells. Meanwhile, tunicamycin treatment induced a 1.8-fold upregulation of transcript 3 (260 bp, supplementary file) in CHO 13D-35D, suggesting that the transcript is involved in UPR. The 5'- and 3'-end sequences of transcript 3 were obtained by the RACE method, resulting in a 1917-bp sequence with initiation and termination codons. The sequence was identified as NF-kappa B inhibitor zeta (NFKBIZ) by a BLAST search and showed 99.8% identity with XM_007622239.2 (NCBI reference sequence, predicted *Cricetulus griseus* Nfkbiz mRNA). Nuclear factor kappa B (NF-κB) proteins are a transcriptional regulator for immune response, apoptosis and proliferation of cells, and their various transcriptional activities are mediated by the inhibitory protein family termed IκB proteins. NFKBIZ (alias IκBζ) is a member of the IκB protein family, and

the physiological functions in the NF- κ B signaling pathway have been studied (Yamazaki et al. 2005; Motoyama et al. 2005; Totzke et al. 2006). Little attention has been paid to NFKBIZ in cell engineering research for recombinant protein production, yet NF- κ B signaling has been reported to be related to gene expression in recombinant CHO cells (Brown et al. 2014, 2015).

Introduction of NFKBIZ into IgG1-producing CHO cells

We introduced the CHO NFKBIZ expression vector into a CHO Hcd6 cell line producing IgG1 antibody to test whether the NFKBIZ works as a production enhancer of recombinant proteins in CHO cells. CHO 13D-35D cell line, the subject of HiCEP analysis, produce antithrombin III, which is composed of a single polypeptide. However, two heavy chains and two light chains are required for IgG1 folding. It is important to evaluate whether NFKBIZ overexpression is effective for IgG1 production, which is more difficult to express. Adherent CHO cells stably expressing NFKBIZ were followed by serum-free adaptation, resulting in two cell lines, NFKBIZ-A and -B. Genomic integration and mRNA expression of introduced NFKBIZ was confirmed by PCR (data not shown). Cell growth, antibody production and quantitative PCR are shown in Fig. 2. A summary of the cell culture performance is given in Table 2. Compared with the mock cell line, each NFKBIZ-overexpressing cell line showed different characteristics of cell growth; maximum viable cell density (VCD) was enhanced in the NFKBIZ-A cell line, whereas that in NFKBIZ-B was reduced. Irrespective of cell growth, IgG1 production in NFKBIZ-B was in equal amounts to that in mock cells. The resulting specific antibody production rate (ρ_{Ab}) in both cell lines was improved by 1.2–1.4-fold compared with the mock cell line (Table 2). In the NFKBIZ-A cell line, the synergistic effect between enhanced VCD and improved ρ_{Ab} brought about a large increase in IgG1 titer of 560 mg/L, while that of mock cells was 360 mg/L. mRNA levels of NFKBIZ, UPR-related genes and IgG heavy- and light-chain at day 7 are shown in Fig. 2c. The NFKBIZ expression level in cell line A was much higher than that in cell line B. Between the mock and NFKBIZ-B, three UPR genes—ATF4, CHOP and GADD34—in the PERK pathway were expressed in

approximately equal amounts. Meanwhile, this expression seemed to be attenuated in NFKBIZ-A. GRP78 expression levels were higher in the two NFKBIZ cell lines. These results suggest that NFKBIZ overexpression may induce GRP78 expression without activating UPR genes in the PERK pathway. No differences were observed in the IgG1 mRNA levels among the cell lines. Although the specific production rate (ρ_{Ab}) was enhanced in both NFKBIZ-A and -B cell lines, the two engineered cells had different characteristics of cell growth and UPR gene expression. One possible explanation is that a threshold expression level is required to enhance cell growth and attenuate UPR gene expression.

NF- κ B binding assay in NFKBIZ-overexpressing cell lines

We investigated the effect of NF- κ B signaling with a luciferase-based reporter system (Fig. 3). NF- κ B is a dimeric complex composed of p50, p52, p65 (RelA), RelB and c-Rel protein (Wietek and O'Neill 2007; Ghosh et al. 2012). The transcriptional activity is regulated by hetero- or homodimerization and its inhibitory protein family I κ B proteins. We focused on two pathways: p50/p65- and p50/p50-dependent transcriptional regulation. NFKBIZ is thought to bind to both p50/p65 and p50/p50 (Yamazaki et al. 2005; Motoyama et al. 2005; Totzke et al. 2006). Although p50/p50 is an inactive form, the homodimer is transformed into active form with transcriptional activity by the binding of NFKBIZ. Meanwhile, the transcriptional activity of p50/p65 heterodimer is inhibited by NFKBIZ binding. Little is known about the molecular mechanism of NFKBIZ-mediated transcriptional regulation, although its C-terminal ankyrin repeat domain is a common binding region for the NF- κ B dimer. We constructed the reporter assay vectors harboring six repeats of each NF- κ B binding nucleotide sequence. The assay vectors were co-transfected into CHO cell lines together with the β -galactosidase vector. A slight variation in transfection efficiency was corrected by β -galactosidase activity. The chemiluminescence intensity of the control vector was at a similar level among the three cell lines. There was also no difference in the intensity from the p50/p65 assay vector. This investigation indicates that p50/p65-heterodimer-mediated NF- κ B signaling was not influenced by NFKBIZ overexpression. Meanwhile, a large

Fig. 2 Effects of NFKBIZ in the recombinant CHO cell line. **a** Viable cell densities. **b** IgG1 production. **c** Relative mRNA levels at day 7. Open, black and gray circles (bars) are mock, NFKBIZ-A and NFKBIZ-B cell lines, respectively. In Fig c, β -Actin was used to normalize the quantification of mRNA

expression. Error bars are 1 S.D. ($n = 3$). Mean values significantly different (two-sided student t test) from the mock control values are indicated by asterisks (* $P < 0.05$, ** $P < 0.01$)

Table 2 Kinetics parameters of cell growth and IgG1 production

Cell	Specific growth rate (μ ; h^{-1})	Specific antibody production rate (ρ_{Ab} ; pg/cell/day)	Relative IgG1 monomer (%)
Mock	0.0152 ± 0.0003	5.2 ± 0.2	94.1 ± 0.1
NFKBIZ-A	$0.0171 \pm 0.0003^{**}$	$7.3 \pm 0.1^{**}$	94.1 ± 0.6
NFKBIZ-B	0.0145 ± 0.0015	$6.4 \pm 0.2^{**}$	95.0 ± 0.1

Mean $\pm$ S.D. ($n = 3$). Mean values significantly different (two-sided student t test) from the mock control values are indicated by asterisks (** $P < 0.01$)

variation was observed in the p50/p50 binding assay vector; a drastic increase in signal intensity was observed in the NFKBIZ-A cell line and a moderate decrease in the NFKBIZ-B cell line. It has been reported that NF- κ B signaling shows the oscillation modes in a manner such that the localization and expression levels of each NF- κ B protein and its

inhibitory I κ B proteins alter its oscillation modes (Nelson et al. 2004; Barken et al. 2005). While the reason for the disagreement in NF- κ B signaling assay results between the two cell lines was not clear, both the constitutive expression of NFKBIZ and the difference in expression levels (Fig. 2c) would induce opposite p50/p50 signaling activity. It has been

Fig. 3 **a** Schematic diagram of the NF-κB binding assay with a luciferase-based reporter system. Six repeats of the p50/p65 and p50/p50 binding sequence were inserted into the forward region of the SV40 promoter of the luciferase expression plasmid. **b** Effects of NFKBIZ overexpression on NF-κB signaling. Open, black and red bars are control, p50/p65 and p50/p50 plasmids, respectively. Error bars are 1 S.D. (n = 3). Mean values significantly different (two-sided student *t* test) from the mock control values are indicated by asterisks (**P* < 0.05, ***P* < 0.01)

reported that the p50/p50/NFKBIZ transcriptional complex is recruited to the promoter region of the interleukin-6 (IL-6) gene (Yamamoto et al. 2004; Motoyama et al. 2005). IL-6 has been known to improve cell growth in B cell hybridoma and mouse plasmacytoma (Van Snick. 1990; Feger et al. 1997). In contrast, suppressed cell growth and improved antibody production after IL-6 addition was reported in a hybridoma cell line (Makishima et al. 1992). There has been no study to our knowledge that showed the endogenous expression of IL-6 in CHO cells. In addition to IL-6 expression, the effects of IL-6 supplementation in recombinant CHO cell culture should be investigated in future work. Irrespective of NF-κB signaling, both NFKBIZ-A and -B cell lines showed enhanced specific IgG1 production rates (Table 2), indicating that enhanced IgG1 productivity is independent of the p50/p50 activating pathway in NF-κB signaling.

Analysis of translational levels and intracellular assembly states of IgG1

We analyzed the translational levels of IgG1 using capillary electrophoresis. Cell lysates from three independent cultures were prepared at day 9, and the lysates were analyzed in reducing and non-reducing conditions. The results of the reducing conditions are shown in Fig. 4a, c. A single band derived from the IgG1 heavy- and light-chain was observed at the corresponding molecular size, and there was no apparent difference in the chemiluminescent signal intensity among the three cell lines, despite the slight decrease in NFKBIZ-B. The results show that NFKBIZ overexpression did not change the transcriptional and translational levels of recombinant IgG1. This is consistent with our previous study regarding the overexpression of ATF4 (Haredy et al. 2013). The results of intracellular folding and assembly are shown in Fig. 4b, d. Analysis under non-reducing conditions enabled us to detect the various assembled states of IgG1: heavy-chain (HC), half antibody (HCLC), heavy chain dimer ((HC)₂), (HC)₂LC and fully assembled IgG1 ((HCLC)₂). In addition to the different assembled states, a fragment with an apparent molecular weight of 96 kDa was also observed, suggesting that an immature IgG1 was subjected to protease digestion in CHO cells. The observed pattern of the assembled states is summarized in Fig. 4d. The overall distributions of all states were similar among the mock and two NFKBIZ-overexpressed cell lines, despite the slight decrease in NFKBIZ-B. (HC)₂ and fully assembled (HCLC)₂ exhibited higher chemiluminescent intensity than did other states, indicating that the dimerization of HC is followed by LC association in the maturation of IgG1. The investigations revealed that unlike with other UPR-based cell engineering, the overexpression of NFKBIZ does not substantially improve the intracellular folding of IgG1. Meanwhile, the specific IgG1 production rate was clearly improved in the two engineered cell lines (Fig. 2; Table 2). Therefore, it was likely that the secretion process or intracellular transport process was improved as a result of NFKBIZ overexpression. In recombinant CHO cells, the IgG polypeptide is mainly localized in the ER, where in some cases correctly folded IgG shows an antibody crystal growth (Hasegawa et al. 2011). In this situation, a large amount of IgG polypeptides must be accumulated in the ER,

Fig. 4 Translational levels and intracellular assembly states of IgG1 at day 9. **a, b** Capillary electrophoresis analysis of IgG1 polypeptides from cell lysates in reducing (**a**) and non-reducing (**b**) conditions. Each of the three lanes of a cell line is a sample from three independent cultures ($n = 3$). **c, d** Chemiluminescence signal intensity estimated from electrophoresis peak

which results in ER overload. It is well known that NF- κ B signaling is activated by ER stresses such as UPR and ER overload (Pahl and Baeuerle, 1997; Ma and Hendershot, 2004). Enhanced secretion and intracellular transport machinery seem to be effective to attenuate ER stress by decreasing IgG1 in the ER. SEC analysis showed a similar elution profile composed of fully assembled monomeric IgG1 among cell lines (Table 1), indicating that the secretion machinery of correctly folded IgG1 was enhanced in NFKBIZ-overexpressing cell lines. The same situation is understood to occur in ATF4 overexpressing CHO cells, resulting in the upregulation of NFKBIZ. While the relation between NFKBIZ overexpression and altered gene expression in NF- κ B signaling remains unresolved, the specific IgG1 production rates were enhanced (Table 2). NFKBIZ-mediated unexplained NF- κ B signaling seems to maintain homeostasis of the

profile in reducing (**a**) and non-reducing (**b**) conditions. Open, black and gray bars are mock, NFKBIZ-A and NFKBIZ-B cell lines, respectively. Error bars are 1 S.D. ($n = 3$). Mean values significantly different (two-sided student t test) from the mock control values are indicated by asterisks (* $P < 0.05$, ** $P < 0.01$)

ER in recombinant CHO cells. In future, the underlying mechanism should be investigated.

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