Overexpression of mutant cell division cycle 25 homolog B (CDC25B) enhances the efficiency of selection in Chinese hamster ovary cells

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Abstract The effects of mutant cell division cycle 25 homolog B (CDC25B) overexpression on the generation of cells producing a monoclonal antibody were investigated in Chinese hamster ovary (CHO) cells. Mutant CDC25B (m-CDC25B) expression plasmids were transfected into CHO DG44-derived cells producing a monoclonal antibody, and the frequency of highly producing cells was assessed following gene amplification in the presence of 250 nM methotrexate. Most of the clones obtained from the m-CDC25B-overexpressing cells had higher antibody titers than did mock-transfected control cells. This arose from either higher transgene copy numbers or higher mRNA expression levels for the antibody. However, the high mRNA expression levels were not always accompanied by increases in transgene copy numbers. Our results suggest that cells producing high levels of a monoclonal antibody can be selected efficiently using m-CDC25B overexpression.

Keywords Chinese hamster ovary (CHO) cells · Gene amplification · Monoclonal antibody · CDC25B

Introduction

Advances in technologies have led to enhancements in the productivity of Chinese hamster ovary (CHO) cells, which are the most popular mammalian host cells in the biopharmaceutical industry (Omasa et al. 2010; Wurm 2004). However, challenges remain in efforts to accelerate the development of suitable cell lines for producing recombinant proteins and in the isolation of highly producing cells, as this usually requires several months (Cacciatore et al. 2010). Dihydrofolate reductase (DHFR) gene amplification, in which a gene of interest is amplified along with a selection marker *dhfr* gene in the presence of methotrexate (MTX), has been used widely to establish productive cell lines (Omasa 2002). Despite its potential to generate highly producing cells, this process is considered as one of the major bottlenecks in the production of recombinant proteins because of the low frequency of such cells, and thus is often not used in the industry (Cacciatore et al. 2012).

We showed previously that gene amplification could be accelerated by the downregulation of a cell cycle checkpoint kinase, ataxia telangiectasia and

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Rad3-related, which increases chromosome instability (Lee et al. 2013a). We also used the overexpression of cell cycle division 25A (CDC25A) phosphatase and found that cell cycle transitions during MTX-induced gene amplification using this approach increased the incidence of cell cycle checkpoint bypass, and highly producing clones could be generated with high frequency through this accelerated gene amplification (Lee et al. 2013b). Cell cycle transition of arrested cells at checkpoints probably increases chromosome instability. Because gene amplification can be initiated by chromosomal breakage (Coquelle et al. 1997), strategies to increase chromosomal instability during gene amplification might be useful tools to generate highly producing clones.

Cell cycle division 25B (CDC25B) is one of the three CDC25 phosphatase isoforms that regulate cell cycle progression. It acts as an important component of checkpoint inhibition and recovery in the event of DNA damage (Boutros et al. 2007; Karlsson-Rosenthal and Millar 2006). CDC25B is primarily responsible for the activation of cyclin-dependent kinase 1 (CDK1) and cyclin B during the G2-M phase transition (Lammer et al. 1998; Lindqvist et al. 2005). It is inactivated by checkpoint kinases 1 and 2 (CHK1 and CHK2) to halt entry to mitosis when DNA is damaged or unreplicated. Deregulation of CDC25B expression resulted in the bypass of cell cycle checkpoints and premature entry into mitosis (Aressy et al. 2008; Bugler et al. 2006). Chromosomal aberrations were also enhanced by CDC25B overexpression in a human cell line (Bugler et al. 2010).

In this study, we investigated whether CDC25B overexpression would accelerate gene amplification and increase the frequency of highly producing clones in CHO cell lines. The effect of CDC25B overexpression on chromosomal aberrations was assessed following MTX-induced gene amplification. The frequency of highly producing clones during gene amplification was evaluated from the level of recombinant antibody produced.

Materials and methods

Cell line and culture

The adherent CHO DG44-derived cell pool (CHO-scDb-Fc) expressing a humanized anti-EGFR $\times$ anti-

CD3 bispecific single-chain diabody with an Fc portion (scDb-Fc) was produced as described (Lee et al. 2013b). All subclones derived from the CHO-scDb-Fc cells were maintained in Iscove's modified Dulbecco's medium (Sigma-Aldrich, St. Louis, MO, USA) containing 10 % dialyzed fetal bovine serum (FBS; SAFC Biosciences, Lenexa, KS, USA) and 500 μ g/mL G418 (Sigma-Aldrich) without hypoxanthine and thymidine at 37 °C under humidified 5 % CO₂ in air. Cells were passaged every 3–4 days into fresh medium at a density of 1×10^5 cells/mL.

Construction of mutant (m) CDC25B expression plasmids

The full-length cDNA of CHO CDC25B was cloned from CHO DG44 cells and fully sequenced. The sequence has been submitted to the DDBJ database (accession number AB841085). The CHO CDC25B cDNA was inserted into *HindIII/XbaI*-digested pcDNA3.1/Hygro(+) expression vectors (Invitrogen Life Technologies, Carlsbad, CA, USA). To construct a mutant CDC25B (m-CDC25B) expression plasmid, site-directed mutagenesis for CDC25B S318A was performed by polymerase chain reaction (PCR) using a PrimeSTAR[®] Mutagenesis Basal Kit (TaKaRa Bio Inc., Otsu, Japan) with the following pairs of primers: 5'-ATCTCCAGCCATGCCGTGCAGTGTGA-3' (forward) and 5'-GGCATGGCTGGAGATCGGAAGAGCCG-3' (reverse). The mutation was confirmed by DNA sequencing. The *Eam1105I*-digested plasmids were transfected into the parental cells using an X-tremeGENE 9 DNA Transfection Reagent (Roche Applied Science, Mannheim, Germany). The stable cells were selected in medium containing 500 μ g/mL G418 (Sigma-Aldrich) and 500 μ g/mL hygromycin (Wako pure chemicals).

Analysis of mRNA expression levels

Total RNA was isolated using a High Pure RNA Isolation Kit (Roche Applied Science) and two-step reverse transcription (RT)-PCR was used for the analysis. A StepOnePlus[™] Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) and a Fast SYBR[®] Green PCR Master Mix (Applied Biosystems) were used for quantification of mRNA expression level with following primers: 5'-CTGGAAGAGCAACAGCCTGA-3' for the CHO *Cdc25B* gene (forward), and

5'-CAATCAGTCCACGGTGGTCA-3' for the CHO *Cdc25B* gene (reverse); 5'-AGGAGTACAAGTGCAA GGTCTCCAAC-3' for the scDb-Fc antibody gene (forward), and 5'-ACCTGGTTCTTGGTCAGCTCAT CC-3' for the scDb-Fc antibody gene (reverse).

The CHO gene for β -actin was used as an internal standard with following primers: 5'-ACTCCTACGT GGGTGACGAG-3' for the CHO gene for β -actin (forward), and 5'-AGGTGTGGTGCCAGATCTTC-3' for the CHO gene for β -actin (reverse).

The following thermal cycling program was applied: 20 s at 95 °C, and 40 cycles of 3 s at 95 °C and 30 s at 60 °C.

Analysis of chromosomal aberrations

CHO cells were treated with colcemid (20 ng/mL) for 4 h, incubated in 75 mM KCl solution for 20 min at room temperature and fixed with Carnoy's fixative (3:1 methanol:acetic acid). The fixed chromosomes were dried using a metaphase spreader (Hanabi, ADSTEC, Funabashi, Japan) and stained with 4',6-diamidino-2-phenylindole (DAPI) for 5 min. Metaphase spreads were scored under an Axioskop 2 fluorescence microscope (Carl Zeiss, Oberkochen, Germany).

Fluorescence in situ hybridization (FISH) analysis

FISH analysis was performed as described (Cao et al. 2012a, b). In brief, the scDb-Fc expression plasmids were labeled using a digoxigenin (DIG)—nick translation mix (Roche Diagnostics, Basel, Switzerland). The labeled-plasmids were hybridized with metaphase chromosome spreads and detected using an anti-DIG rhodamine-labeled antibody (Roche Diagnostics). Metaphase chromosome spreads were counterstained with DAPI and observed under an Axioskop 2 fluorescence microscope (Carl Zeiss).

Analysis of gene copy number

The transgene copy number was determined by quantitative real-time PCR as described (Lee et al. 2013b). Briefly, genomic DNA was isolated using a High Pure PCR Template Preparation Kit (Roche Applied Science) and RNase A (Sigma-Aldrich). The CHO gene for β -actin was used as an internal control and the PCR reaction was performed using a Fast

SYBR® Green PCR Master Mix (Applied Biosystems) with following primers: 5'-AGGAGTACAAGT GCAAGGTCTCCAAC-3' for the scDb-Fc antibody gene (forward), and 5'-ACCTGGTTCTTGGTCAG CTCATCC-3' for the scDb-Fc antibody gene (reverse); 5'-GCTGTGGGTGTAGGTACTAACA T-3' for the CHO gene for β -actin (forward), and 5'-GAATACACACTCCAAGGCCACTTA-3' for the CHO gene for β -actin gene (reverse). The following thermal cycling program was applied: 20 s at 95 °C, and 40 cycles of 3 s at 95 °C and 30 s at 60 °C. Standard curves were generated using serial dilutions of the antibody expression plasmids and β -actin fragments.

Antibody titer analysis

Secreted antibody concentrations were quantified by a standard sandwich enzyme-linked immunosorbent assay as described (Kim et al. 2010; Onitsuka et al. 2012). A standard curve was generated using purified human immunoglobulin G (Athens Research & Technology, Athens, GA, USA). The assay was performed in triplicate for each sample.

Results

Construction of m-CDC25B-overexpressing cells

Thirty-four clones were isolated from the CHO-scDb-Fc cell pool using a limiting dilution method. Among these, CHO clones designated SD-S23 and SD-S25 were selected for further experiments on the basis of productivity. The activity of CDC25B is regulated by the proteasome-dependent pathway and its cellular localization (Kieffer et al. 2007). Serine 323 (S323) in human CDC25B is known as a major phosphorylation site that influences CDC25B degradation and localization. The mutation of S323 site of human CDC25B prevented its cytoplasmic localization and degradation (Davezac et al. 2000; Giles et al. 2003). In addition, the overexpression effect of S323-mutated CDC25B on the initiation of aberrant mitosis was higher than that of wild-type CDC25B (Forrest and Gabrielli 2001). In this study, serine 318 of CHO CDC25B—equivalent to S323 of human CDC25B—was substituted with alanine to enhance its stability and increase genomic instability.

Fig. 1 Quantitative real-time PCR analysis for CDC25B mRNA expression levels in **a** CHO SD-S23 cells, **b** CHO SD-S25 cells, and **c** CHO-scDb-Fc cells. The CHO gene for β -actin was used as

an internal standard. The error bars represent the standard deviation ($n = 3$). ** $P < 0.01$ versus control by two-tailed unpaired Student's t tests

The m-CDC25B expression plasmids were transfected into the SD-S23, SD-S25 and CHO-scDb-Fc cells. The pcDNA3.1/Hygro(+) null plasmid was used to generate control cells. To confirm CDC25B overexpression, the CDC25B mRNA expression levels in stable transfectants were investigated by quantitative real-time PCR. As shown in Fig. 1, the stable CHO SD-S23 and SD-S25 transfectants showed 1.6-fold higher CDC25B mRNA expression levels compared with the mock-transfected cells. The CHO-scDb-Fc cell pool also showed a 2.4-fold higher CDC25B mRNA expression level than the mock-transfected cells.

Increase in chromosomal aberrations by m-CDC25B overexpression

We investigated the effect of m-CDC25B overexpression on chromosomal aberrations such as gaps or breaks in metaphase chromosome spreads. To observe chromosomal aberrations in the early stages of MTX-induced gene amplification, CHO-scDb-Fc cells overexpressing m-CDC25B and mock-transfected cells were cultured for 45 h in the presence of 250 nM MTX and arrested in M phase by 20 ng/mL colcemid treatment for 4 h. We examined 100 chromosome spreads in each cell pool ($n = 3$) by direct counting of chromosomal aberrations. As shown in Fig. 2, m-CDC25B overexpression caused a significant increase in chromosomal aberrations. An average of 19 individual cells showed gaps or breaks in the cell pool overexpressing m-CDC25B, a 2.4-fold higher frequency compared with an average of eight cells in

the mock-transfected pool. Moreover, aberrant cells in the m-CDC25B-overexpressing cells had an average of 2.3 gaps and breaks, whereas the mock-transfected cells had an average of 1.5 gaps and breaks per cell. Bugler et al. (2010) showed that elevated levels of CDC25B could cause chromosome instability in human U2OS cells. Our results confirmed that m-CDC25B overexpression increased chromosomal aberrations in CHO cells. These data suggest that although MTX treatment alone can cause chromosomal gaps or breaks during gene amplification, the frequency of chromosomal aberrations could be increased significantly by m-CDC25B overexpression.

Frequency of highly producing clones during gene amplification

We hypothesized that highly producing clones could be generated in a cell pool more efficiently via accelerated gene amplification by m-CDC25B overexpression. To test this hypothesis, we carried out gene amplification using CHO SD-S23 and SD-S25 cells overexpressing m-CDC25B in the presence of 250 nM MTX. Fifty single clones were isolated by limiting dilution in 96-well plates, and 10 highly producing clones in each cell pool were transferred into 24-well plates for productivity analysis. The single clones were inoculated into 24-well plates at a concentration of 1×10^5 cells/well in 0.8 mL medium, and antibody titers in the supernatants were analyzed after 4 days of culture. As shown in Fig. 3, the single clones in the CHO SD-S23 cell pool overexpressing m-CDC25B

Fig. 2 Analysis of metaphase chromosomal aberrations during gene amplification. **a** Representative chromosomal aberrations in CHO-scDb-Fc cells overexpressing m-CDC25B in the presence of 250 nM MTX. The CHO-scDb-Fc cells were cultured for 45 h in the presence of 250 nM MTX and metaphase chromosome spreads were prepared using colcemid treatment. *Arrows* indicate the chromosomal aberrations. **b** Aberrant cell numbers in 100

metaphase chromosome sets. **c** Average chromosomal aberrations per aberrant cell. Chromosomal aberrations were counted directly under a fluorescence microscope. The *error bars* represent the standard deviation of three independent experiments. ** $P < 0.01$ and * $P < 0.05$ versus control by two-tailed unpaired Student's *t* tests

had higher antibody titers than the mock-transfected cells. Although the antibody titers of single clones in the mock-transfected pool ranged from 1.19 to 4.39 $\mu\text{g/mL}$, only one clone had a titer over 2 $\mu\text{g/mL}$. Meanwhile, all single clones in the m-CDC25B-overexpressing cell pool had titers higher than 2 $\mu\text{g/mL}$. Single clones in the CHO SD-S25 cell pool overexpressing m-CDC25B also showed higher titers (1.17–3.65 $\mu\text{g/mL}$) than did the mock-transfected cells (0.03–1.34 $\mu\text{g/mL}$). These results suggest that m-CDC25B overexpression can generate highly producing clones in a cell pool during gene amplification.

Estimation of transgene copy number

To investigate whether m-CDC25B overexpression induced an increase in transgene copy numbers,

genomic DNAs were extracted from five highly producing clones in each cell pool selected in the presence of 250 nM MTX, and scDb-Fc antibody gene copy numbers were analyzed by quantitative real-time PCR. In the m-CDC25B-overexpressing CHO SD-S23 cell pool, single clones had 10.16 ± 0.54 to 14.23 ± 1.02 scDb-Fc antibody gene copies per β -actin gene, higher than those of single clones in the mock-transfected pool (0.57 ± 0.03 to 8.98 ± 0.74 copies) (Fig. 4). However, the transgene copy numbers of single clones in the m-CDC25B-overexpressing CHO SD-S25 cell pool (28.72 ± 1.24 to 42.32 ± 2.76) were not higher than those of the mock-transfected pool cells (31.34 ± 3.83 to 61.03 ± 2.73). These results were confirmed by FISH analysis. As shown in Fig. 5, fluorescence signal patterns of the m-CDC25B-overexpressing CHO SD-S23 single clones were much

Fig. 3 Antibody titers of 10 highly producing clones in each cell pool in the presence of 250 nM MTX. Antibody titers were determined on day 4 in a 24-well plate culture. **a** SD-S23 cells. **b** SD-S25 cells. Gray bars show single clones in the mock-

transfected pool; black bars show single clones in the m-CDC25B-overexpressing cell pool. The error bars represent the standard deviation ($n = 3$)

broader and stronger than those of the mock-transfected clones. We also observed that single clones in the m-CDC25B-overexpressing CHO SD-S25 cell pool had similar fluorescence signal intensities to the mock-transfected clones, which are consistent with the transgene copy numbers calculated by quantitative real-time PCR.

Comparison of scDb-Fc mRNA expression levels

The antibody mRNA expression levels were assessed by quantitative real-time PCR. In the CHO SD-S23 cells, although the mock-transfected clone CHO S23-M17 had the highest mRNA expression level, the remaining clones in the mock-transfected pool had much lower mRNA levels than in the m-CDC25B-overexpressing

cell pool. The mRNA expression levels of single clones in the m-CDC25B-overexpressing cell pool were up to eightfold higher than in the CHO S23-M16 clone having the lowest mRNA level in the mock-transfected cells (Fig. 6). Whereas the antibody mRNA expression levels in the SD-S23 cell pool were almost proportional to the transgene copy numbers, those in the CHO SD-S25 cell pool were not consistent with the gene copy numbers. Although the single clones in the m-CDC25B-overexpressing CHO SD-S25 cell pool had similar or lower copy numbers compared with the mock-transfected clones, their mRNA expression levels were much higher, showing up to 19-fold higher mRNA levels. Interestingly, the CHO S25-B40 clone showed the highest mRNA expression level despite having the lowest copy number among the single clones in the m-CDC25B-overexpressing CHO SD-S25 cells.

Fig. 4 Analysis of relative transgene copy number. The scDb-Fc antibody gene copy number relative to the gene for β -actin of five highly producing clones in each cell pool was determined by quantitative real-time PCR. **a** CHO SD-S23 cells. **b** CHO SD-S25 cells. Gray bars show single clones in the mock-transfected pool; black bars show single clones in the m-CDC25B-overexpressing cell pool. The error bars represent the standard deviation ($n = 3$)

Discussion

The DHFR-mediated gene amplification system is considered a time-consuming and labor-intensive process. To enhance the efficiency of this system, many researchers have developed novel methods with which highly producing cells can be more stringently selected (Cacciatore et al. 2010). A short hairpin RNA (shRNA) vector targeted to the *dhfr* gene showed a productivity increase in the transgene-amplified stable CHO cells (Wu et al. 2008). The improvements in productivities using mRNA and protein destabilizing elements with *dhfr* gene were also reported (Ng et al. 2007). The strategy of both methods is to reduce *dhfr* gene expression or protein products for the selection of highly producing cells. The use of dual selection marker is another approach to improve gene amplification efficiency (Peroni et al. 2002). We previously

proposed novel methods to enhance the efficiency of the standard gene amplification system using cell cycle checkpoint engineering. In this study, we have examined the effects of m-CDC25B overexpression on gene amplification efficiency.

Overexpression of m-CDC25B increased the rate of chromosomal aberrations markedly during MTX-induced gene amplification. Because the efficiency of gene amplification was associated with an increased incidence of chromosomal aberrations in our previous study (Lee et al. 2013a), we expected that highly producing cells would be more efficiently generated by m-CDC25B overexpression via accelerated gene amplification. The antibody titers of the selected clones in the m-CDC25B-overexpressing CHO SD-S23 and S25 cells were much higher than the mock-transfected cells, showing many highly producing cells were generated by m-CDC25B overexpression. Interestingly, analysis of their molecular features revealed that these improvements in productivity were not always accompanied by an increase in transgene copy numbers. While the CHO SD-S23 cells overexpressing m-CDC25B had both more gene copies and increased mRNA expression levels compared with the mock-transfected cells, the SD-S25 cells overexpressing m-CDC25B showed higher mRNA expression levels with similar or less gene copies compared with the mock-transfected cells. We surmise that m-CDC25B overexpression causing chromosome instability might act as a stress threatening cell survival during MTX-induced gene amplification and that this increases the selection pressure more than the standard method. In such conditions, those cells that can overcome the strong selection pressure by increases in transgene copy numbers or mRNA expression levels might survive, resulting in high levels of antibody production. In our previous research, we showed that CDC25A overexpression contributes to increasing the efficiency of gene amplification (Lee et al. 2013b). Although both CDC25A and CDC25B are involved in G2-M transition and their functional differences in mitosis remain elusive, some researchers reported that CDC25A has a role in the initiation of chromatin condensation and CDC25B is involved in the activation of CDK1 and cyclin B at the centrosomes (Boutros et al. 2007; Lindqvist et al. 2005). We revealed that high-producing cells could be efficiently selected by the overexpression of either CDC25A or m-CDC25B during

Fig. 5 FISH analysis of metaphase chromosomes. Metaphase chromosomes were prepared after gene amplification in the presence of 250 nM MTX. *Arrows* indicate the amplified genes. **a** CHO SD-S23 mock-transfected cell. **b** CHO SD-S23 cell overexpressing m-CDC25B. **c** CHO SD-S25 mock-transfected cell. **d** CHO SD-S25 cell overexpressing m-CDC25B

gene amplification process. However, further study for the relationship between their specific roles in mitosis and gene amplification might be useful for industrial applications.

We used CHO SD-S23 and SD-S25 as two parental cell lines and observed that the antibody-expressing plasmids were integrated into the middle of the p-arm of a chromosome in CHO SD-S25 cells and into the subtelomeric region of the q-arm of another chromosome in CHO SD-S23 cells. The antibody titers of clones selected from CHO SD-S23 cells were clearly correlated with mRNA expression levels, which in turn were also almost proportional to the transgene copy number. Notably, even though single clones from CHO SD-S23 cells had lower transgene copy numbers than the CHO S25B-40 clone with the lowest copy number in the CHO SD-S25 cells, their antibody titers were higher. Chromosomal position is well known to influence the efficiency of transgene transcription (Wilson et al. 1990). Our results suggest that the plasmid integration site in CHO SD-S23 cells was probably in a transcriptionally active region. By contrast, the antibody expression plasmid might have been integrated into a transcriptionally inactive region in the CHO SD-S25 cells, as there was no clear correlation between the mRNA expression levels and

transgene copy number in these cells. Although the single clones in the CHO SD-S25 cell pool overexpressing m-CDC25B had similar or lower transgene copy numbers compared with the mock-transfected cells, their mRNA expression levels were much higher, resulting in higher antibody titers. We could not elucidate whether m-CDC25B overexpression acts only to strengthen selection pressures or whether it prevents amplified transgene silencing in the gene integration sites. Further study is required to elucidate the mechanism of selection of highly producing clones in CHO SD-S25 cells by m-CDC25B overexpression. In addition, overexpression of m-CDC25B in an inducible manner during gene amplification process should also be considered for industrial applications. Although an increase in chromosomal instability by m-CDC25B overexpression could enhance the selection efficiency, its constitutive expression might be harmful to cells. A conditional expression of m-CDC25B using Tet-On/Off systems might be suitable to maintain chromosomal stability after cell line construction.

In this study, we demonstrated that m-CDC25B overexpression enhanced the efficiency of the standard gene amplification system. Cells producing high levels of antibodies were more efficiently selected

Fig. 6 Comparison of relative mRNA expression levels for the scDb-Fc antibody gene. Relative mRNA expression levels of five highly producing clones in each cell pool were analyzed by quantitative real-time PCR. The CHO gene for β -actin was used as an internal standard. **a** CHO SD-S23 cells. **b** CHO SD-S25 cells. *Gray bars* show single clones in the mock-transfected pool; *black bars* show single clones in the m-CDC25B-overexpressing cell pool. The *error bars* represent the standard deviation ($n = 3$)

by m-CDC25B overexpression during MTX-induced gene amplification. This approach could help accelerate cell line development by increasing the possibility of isolating such productive cell lines.

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