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Artificial transcription factors increase production of recombinant antibodies in Chinese hamster ovary cells

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Abstract

A randomized library that encodes for artificial zinc finger protein transcription factors (ZFP-TF) was constructed and screened for components that increased production of a monoclonal antibody (mAb-72) in Chinese hamster ovary (CHO) cells. One of these ZFP-TF, LK52, increased mAb-72 production ~10-fold at ~60% transduction efficiency; a mutated version of LK52, however, did not boost mAb-72 production. LK52 also increased production of other mAbs in CHO cells. These results demonstrate that ZFP-TF libraries can be used to identify components that improve antibody production in CHO cells.

Introduction

The potential of therapeutic monoclonal antibodies (mAbs) to serve as medicinal agents is rapidly increasing. Chinese hamster ovary (CHO) cells are often used for their production on a large scale (Andersen & Krummen 2002). A current goal is therefore to increase the yield of antibodies from CHO cells.

A variety of methods have been assessed in the quest for enhanced production of all types of recombinant proteins, including mAbs. The first approach was the development of expression vectors that greatly enhance transcription of the gene of interest (Kim et al. 2002, Kwaks *et al.* 2003). Another method is to modify the host cell line by either (i) the co-expression of chaperones (Mosser & Massie 1994); (ii) inhibition of apoptosis (Vives *et al.* 2003); or (iii) induction of resistance to certain stresses (for example, osmolarity) (Kim *et al.* 2000). A third technique is to optimize cell growth and division (Altamirano *et al.* 2000). The first approach requires the characterization of transcriptional regulatory sequences, while the second approach requires knowledge of the cell's limiting factor for protein production. Such extensive characterization can be difficult and time consuming.

The Cys_2His_2 zinc finger domain (ZFD) is a DNA binding domain. Individual ZFDs function as modules and exhibit binding to diverse and predictable DNA sequences (Wolfe *et al.* 2000, Beerli

& Barbas 2002). Transcription factors for ZEPs (ZFP-TF) are constructed by fusing together several ZFDs in a given order and adhering this newly created, multi-finger domain to one of a variety of transcriptional activation or repression domains. ZFP-TFs have been used to modulate, at will, the expression of genes that yield desired phenotypes in mammalian, plant, and bacterial cells (Joung *et al*. 2000, Rebar *et al*. 2002, Sanchez *et al*. 2002, Blancafort *et al*. 2003, Park *et al*. 2003, Lee *et al*. 2004). If the genome sequence of a test organism is known, one can use ZFP-TF library screening and genome-wide gene expression profiling to identify genes that regulate the manifestation of specific phenotypes of interest (Park *et al*. 2003). Here, we used the randomized ZFP-TF library approach to improve antibody production in CHO cell lines.

Materials and methods

Cell lines

The AKA cell line, a derivative of a CHO cell line that produces monoclonal antibody-72 (mAb-72) [which is a humanized version of the TAG72 (tumor associated glycoprotein 72) antibody], was obtained from Dr. H. J. Hong at the Korean Research Institute of Bioscience and Biotechnology. AKA cells were grown in α-minimal essential media (α-MEM) (JBI, Korea) that contained 10% (v/v) dialyzed fetal bovine serum (FBS; JBI). Human embryonic kidney (HEK) 293T cells (ATCC No. CRL-11268) were grown in Dulbecco's modified Eagle medium (DMEM) (JBI) containing 10% (v/v) heat-inactivated FBS. The SH2-0.32 and SO-0.32 cell lines were constructed as described previously (Kim *et al*. 1998, 2001) and were grown in α-MEM or Iscove's modified Dulbecco's medium (IMDM) (JBI) that contained 10% (v/v) dialyzed FBS and either 320 or 80 nM methotrexate (MTX), respectively.

Construction of expression vectors

The LNCX expression vector (Clontech, USA) was used for ZFP-TF delivery into cultured cells. A modified version of LNCX, LNCX2, was used to clone a DNA fragment that contained a transcription initiation codon as well as sequences that encode the SV40 nuclear localization signal and a transcriptional repression (KRAB, NCBI accession number M96548) or activation (VP16, NCBI accession number X03141) domain. A library of the DNA fragments that encoded ZFPs that consisted of four ZFDs (Park *et al*. 2003) was cloned into LNCX2-KRAB or LNCX2-VP16, resulting in LNCX2-ZFP-KRAB or LNCX2-ZFP-VP16, respectively.

The LNCX2-ZFP-IRES-GFP family of vectors was constructed by inserting, downstream of the ZFP-KRAB coding region in LNCX2-ZFP-KRAB, a DNA fragment [from pIRES-hGFP-1a (Stratagene, USA)] that encodes an internal ribosome entry site and the green fluorescent protein (IRES-GFP). With this expression vector, various ZFPs and GFP could be transcribed as parts of the same mRNA molecule.

To obliterate the binding activity of LK52-KRAB, we substituted the two arginine residues in the RDHT and QSSR1 ZFDs of LK52 – amino acids critical for the ZFP's specific DNA binding activity – with alanine residues using PCR-mediated site-directed mutagenesis.

Preparation and transduction of retrovirus particles

To prepare virus particles that contained ZFP-encoding genes, HEK 293T cells were seeded in 96- or 24-well plates at 6×10^4 or 3×10^5 cells/well, respectively. Transient transfections were carried out with Lipofectamine 2000 (Invitrogen) according to the manufacture's instructions. For each well of cells in the 96-well plates, 100 ng of the LNCX2-ZFP-TF plasmids were co-transfected with 50 ng of pGag-Pol (Takara, Japan) and pVpack-VSV-G (Stratagen); cells in the 24-well plates were co-transfected with four times the amounts of the same plasmids. The cells were incubated for 6 h after transfection, and then the medium was replaced with fresh medium containing 10 mM sodium butyrate. The medium was replaced again after a further 16 h incubation, and then medium containing viral particles was harvested after an additional 24 h incubation.

For viral infection, AKA cells were plated in 96- or 24-well plates at a density of 1.5×10^4 or 3×10^4 cells/well, respectively. After 1 day of incubation, 50 or 250 μl of an infection cocktail

[a mixture of retroviral particles and fresh medium mixed at a ratio of 1:1 (v/v)] was added to each well of the 96- or 24-well plates with 0.4 or 2 μg of polybrene, respectively. The cell supernatant was replaced with fresh medium the next day. After further incubation for the indicated times, the medium was harvested for measurement of mAb production by ELISA. For continuous assessment of mAb production, 200 μl medium from each well of the 24-well plates was harvested daily for 6 days and frozen for future ELISA. The ELISAs were conducted as described previously (Kim *et al*. 1996). To measure transduction efficiency, the LNCX2-ZFP-KRAB-IRES-GFP expression vectors were used for viral preparation and transduction. Fluorescent-activated cell sorting (FACS) analysis was performed as described previously on a FACSCalibur 110VAC (Becton Dickinson, U.S.A) at 488 nm. For screening purposes, we tested about 1500 ZFP-KRABs and 500 ZFP-VP16s in 96-well plates. After ELISA and data analysis, ZFP-TFs that were related to increased production of mAb were retested to confirm the results.

Results

Screening of a randomized ZFP-TF library and identification of ZFP activators

To select for a ZFP-TF that increases antibody production in CHO cells, we used AKA cells, a CHO cell line that expresses a mAb directed against the TAG72 protein (mAb−72). We employed a retroviral vector system to deliver ZFP-TFs into cells, because it can transfer the gene to more cells at a lower copy number than is possible with transient transfection. Individual plasmids were isolated from a LNCX2-ZFP-KRAB or -VP16 library and used, along with 293T cells cultured in 96-well plates, to prepare retroviral particles that each contained a ZFP-TF expression plasmid. The virus particles were transduced into AKA cells seeded in 96-well plates, and, after the indicated time of incubation, ELISAs were carried out to measure the amount of secreted mAb. After screening 2000 ZFP-TFs, we isolated the four candidates that yielded the largest increases in mAb-72 production. All 4 ZFP-TFs contained the inhibitory KRAB segment as their functional domain. This finding indicates that these ZFP-TFs increased mAb-72 production, not by direct activation of the mAb-encoding gene, but by an indirect mechanism that involved the repression of target gene transcription. The identities and putative target DNA binding sites of these 4 ZFP-TFs are shown in Table 1 (LK33, LK35, LK50, and LK52).

The four candidate ZFP-TFs were individually transduced into AKA cells as virus particles, and the cells were incubated for two days, after which we measured a 2- to 6-fold increase in mAb-72 production, compared to an empty vector control or a vector harboring only the KRAB domain (Figure 1). To rule out the possibility that the observed increase in mAb-72 production resulted from an increase in the number of cells, we carried out an MTT assay for cell proliferation and observed no obvious difference in cell numbers among the 6 samples referred to in Figure 1 (data not shown).

It is important to note that the increase in mAb-72 production that we measured for the 4 ZFP-TFs referred to in Figure 1 might be an underestimate. This is because the ZFP-TFs were not delivered to 100% of the cells. In reality, the efficiency of delivery was 40–80%. Therefore, if we assume 50% efficiency, the observed 2-fold increase in activity is actually a 3-fold increase $[(3\times0.5)+0.5=2]$. To measure transduction efficiencies more accurately, we constructed plasmids

Table 1. Identities of zinc finger proteins (ZFPs) of interest and their putative DNA binding sites.

ZFP name	Finger 1	Finger 2	Finger 3	Finger 4	Putative target sequence (5′–3′)
LK33	RDHT	QTHR1	QSNK	QSSR1	GYADAAVGANGG
LK35	RDHT	QSNR1	QTHR1	QSNI	MAAVGAGAANGG
LK50	QTHR1	RDKR	RDHT	QTHR1	VGANGGRGGVGA
LK52	RDHT	QSHV	QTHQ	QSSR1	GYAHGAHGANGG

Y:C or T; D: A, G or T; V: A,C or G; N: A,C, G or T; M: A or C; R: A or G; H: A, C or T.

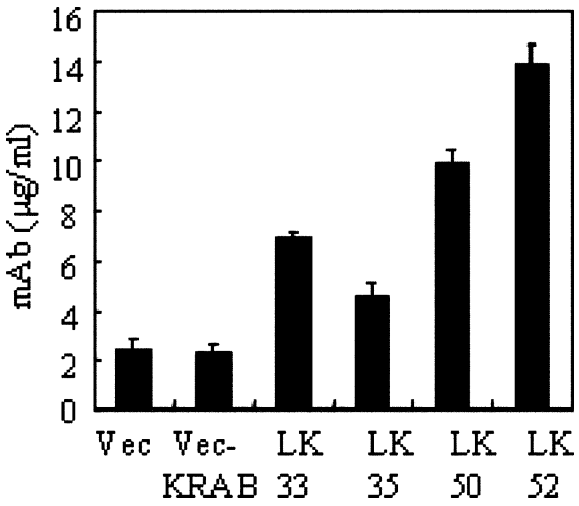

Fig. 1. Increased production of mAb in AKA cells transduced by viruses containing one of 4 ZFP-TFs. Virus production, transfections, and ELISAs were performed as described in Materials and methods. Vec and Vec-KRAB, respectively, indicate the control (empty) LNCX2 vector and the LNCX2 vector with only the KRAB repression domain.

that contained genes for either the LK50 or LK52 ZFP-TF and GFP, connected by sequences that encoded an internal ribosome entry site (IRES). This was done so that the ZFP-TFs and GFP could be translated from the same mRNA transcript and, thus, transduction efficiency could be measured by FACS analysis of the cellular GFP expression level.

Viral particles that contained the corresponding ZFP-IRES-GFP constructs were transduced into AKA cells as described above, and their fold activities and transduction efficiencies were measured by ELISA and FACS analysis, respectively. The transduction efficiencies were ~40–80%, and the mAb−72 amounts produced were increased 4- to 8-fold (Figure 2). These data suggest that the selected ZFPs could make AKA cells produce more mAb-72 if the ZFP-TF expression vectors were delivered into cells with 100% efficiency. For example, LK52 has the potential to boost mAB-72 production ~10-fold. The discrepancy between the amounts of antibody observed in Figure 1 versus Figure 2 might result from changes in cell physiology, differences in the transduction efficiency of each sample, or unknown effects of the IRES and/or GFP.

LK52-related increase in antibody production by AKA and other antibody-producing cells

Because LK52 exhibited the highest transduction efficiency and mAb-72 production among the 4 ZFP-TFs selected, we turned our efforts toward further characterization of this protein. To test whether the observed increase in mAb−72 production resulted from LK52's ability to bind DNA and modulate the transcription of target genes, we generated a mutated version of LK52 that was not able to bind its putative target DNA binding site. Retrovirus particles that contained either the wild type LK52 expression vector, the mutant LK52 expression vector, or a control vector were transduced into AKA cells, and the

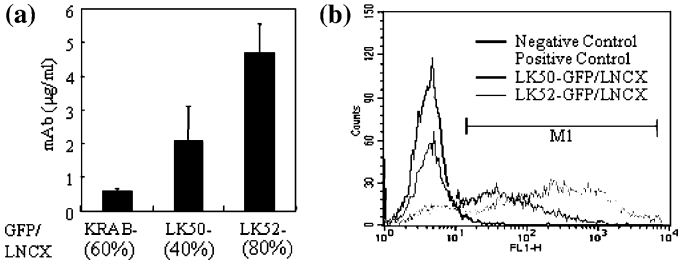

Fig. 2. (a) mAb production in and viral transduction efficiency of cells carrying LK50 or LK52. To measure the transduction efficiencies of virus carrying one of each of the two ZFP-TFs, LK50 and LK52, the LNCX2-LK50 or -LK52 was fused to the IRES-GFP fragment, resulting in the corresponding LNCX2-ZFP-IRES-GFP plasmid. Preparation and transduction of virus were done using these plasmids. ELISAs were carried out as described in Materials and methods, except that the cells were seeded in 24-well plates. The transduction efficiency of each sample was measured and shown in parentheses. (b) A FACS analysis of GFP expressed by the cells described in (a). The transduction efficiencies of samples were estimated by comparison of the percentage of experimental AKA cells (those transduced with each of the indicated plasmids) present in the M1 region (an amount of >10 for fluorescence on the X-axis) compared with the percentage of control AKA cells (non-transduced; negative control, black line) present in the M1 region. LK50 (blue line) and LK52 (red line) are shown with a control containing only the KRAB domain (green line). Y and X axes indicate cell counts and fluorescence level, respectively.

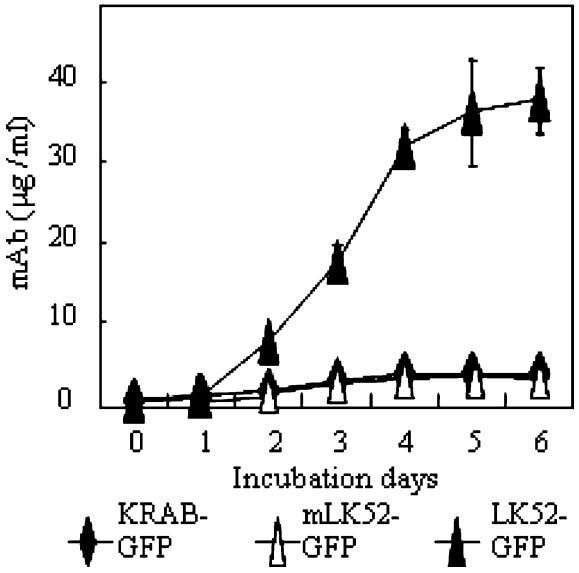

Fig. 3. mAb accumulation pattern in AKA cells transduced with an LK52 expression vector (closed triangles), a control vector (closed diamonds), or a mutated LK52 (open triangles) vector. LNCX2 plasmids encoding LK52 or mutated LK52 were transfected into HEK 293T cells, and experiments were carried out as described in Materials and methods, except that the cells were grown in 24-well plates. Following viral transduction (Day 0), the culture media was changed after incubation for one day. For the next 5 days (days 2–6), the same volume of culture medium was removed and frozen. On day 7, all frozen samples, including the day 0 and day 1 samples, were subjected to ELISA. The empty LKCX2 vector containing only the KRAB domain (KRAB) was also tested in this series of experiments. The viral transduction efficiency was about 50% for all three samples, as measured by FACS analysis.

amount of accumulated mAb−72 in each sample was measured each day for 6 days (Figure 3). Cells containing wild type LK52 accumulated mAb-72 continuously over the 6-day period up to a maximum of 37 μg/ml, whereas cells that contained the control vector exhibited little increase in mAb accumulation over the same time period (the increase in LK52-related mAb production was ~10-fold compared to the control). The mutated version of LK52 showed a production pattern similar to that of the control (Figure 3), which suggests that the observed increase in LK52-related mAb-72 production was dependent on the binding of LK52 to its target genes.

To investigate whether LK52 could increase production of other kinds of antibodies in cultured cells that were not used for ZFP-TF library screening, we chose two CHO cell lines in which amplification of the *mAb* gene was induced by treatment of the cells with MTX. These cell lines, SH2-0.32 and SO-0.32, produce antibodies to the S surface antigen of the hepatitis B virus and the glycoprotein receptor of platelets, respectively, and their accumulated antibody concentrations were 9 μg/ml (S surface antigen mAb) and 75 μg/ml (glycoprotein receptor mAb) when measured after 3 days of incubation. As shown in Figure 4, LK52 increased mAb production approximately 4- and 1.7-fold in SH2-0.32 and SO-0.32 cell lines, respectively, compared to empty vector controls (Vec and Vec-KRAB), which showed a level of production similar to that of cells subjected to mock transduction (data not shown). Clearly the fold increases in mAb production by LK52 in the SH2-0.32 and SO-0.32 cell lines were smaller than that observed in AKA cells. It is possible that the fold increases would be enhanced if ZFP-TFs were isolated by screening in these specific cell lines. Nevertheless, these increases are significant and thus may be useful in the context of mass production.

Discussion

The limits imposed by facilities in the production of recombinant proteins have become one of the key obstacles in the protein therapeutics field. Several reports have shown that one can increase the production of recombinant proteins through the regulation of specific target genes in mammalian

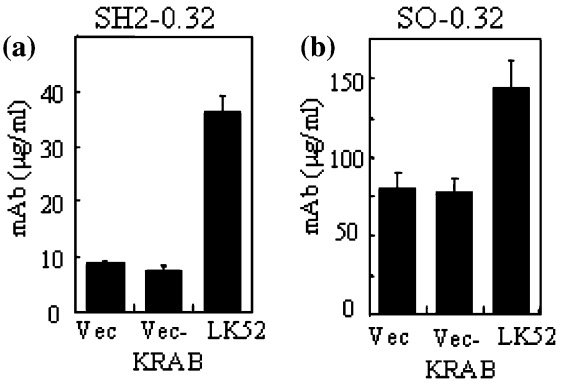

Fig. 4. Effect of LK52 on the production of two different mAbs in two additional CHO cell lines. Vec and Vec-KRAB indicate the LNCX2 vector and the LNCX2 vector with the KRAB repression domain, respectively.

cells (Kim & Lee 2000, 2001) as well as in *E. coli* (Han *et al*. 2003). Here, using a randomized ZFP-TF library in CHO cells, we have successfully extended this approach to include the regulation of unknown target genes. The ZFP-TF library approach does not require the identification of a suitable target gene for its application. In addition, the isolated ZFP-TFs could be used to identify the target genes when coupled with a genome-wide gene expression profiling approach. This series of methods has been successfully applied to yeast (Park *et al*. 2003), but cannot yet be used with CHO cells for which no cDNA microarray chip is commercially available. Once microarray experiments are possible for CHO cells, novel target genes that facilitate recombinant protein production can be identified.

The amount of mAb−72 produced by AKA cells that had been transduced with LK52 (37 μg/ml) may be regarded as too low when compared with the amounts of the mAb generated by commercial mAb-producing cell lines. However, the AKA cell line was selected after only one cycle of MTX amplification and was cultured as adhesive cells with no medium change during the measurement of mAb production. Therefore, this 10-fold increase brought about by LK52 in the AKA cells might represent an important improvement. In addition, we have shown that the same ZFP-TF could be applied to increase the mAb productivity of other cell lines. This observation suggests that one active ZFP-TF can be used to boost the production of a wide range of antibodies in a number of mAb-producing cell lines.

The isolated ZFP-TFs can be used in two ways. The first is to stably transfect a CHO cell line, such as DG44 or DUKX-B11, with genes that encode one of the selected ZFP-TFs and to use this cell line as a host for amplification of the gene(s) of interest. The second is to express the ZFP-TF as a fusion to a protein transduction domain (PTD) (Green & Loewenstein 1988, Frankel & Pabo 1988). PTD is a small peptide domain that can enter cells when added to the culture medium, perhaps by endocytosis (Becker-Hapak *et al*. 2001). Of the several known PTDs, that of the TAT protein (Fawell *et al*. 1994) has been studied most intensively. Proteins such as β-galactosidase could be transduced into cells and have been shown to function normally when fused to a TAT PTD (Fawell *et al*. 1994). If a TAT-LK52 fusion protein functions in a way similar to an *LK52* gene that has been transduced into cells via virus particles, one can imagine that addition of an LK52-PTD fusion protein to the medium might increase the production of heterologous proteins.

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