ARTICLE

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A DIGE Approach for the Assessment of Differential Expression of the CHO Proteome Under Sodium Butyrate Addition: Effect of Bcl-x_L Overexpression

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ABSTRACT: Bcl-x_L, a member of the Bcl-2 family, is known to inhibit apoptosis of recombinant Chinese hamster ovary (rCHO) cells induced by the addition of sodium butyrate (NaBu), which is used for the elevated expression of recombinant protein. In order to understand the intracellular effects of Bcl-x_L overexpression on CHO cells treated with NaBu, changes to the proteome caused by controlled Bcl-x_L expression in rCHO cells producing erythropoietin (EPO) in the presence of 3 mM NaBu were evaluated using two-dimensional differential in-gel electrophoresis (2D-DIGE) and MS analysis. The consequences of Bcl-x_L overexpression were not limited to the apoptotic signaling pathway. Out of eight proteins regulated significantly by Bcl-x_L overexpression in 3 mM NaBu addition culture, four proteins were related to cell survival (Iq motif-containing GTPase-activating protein 1), cell proliferation (dihydrolipoamide-*S*-acetyltransferase, guanine nucleotide binding protein alpha interacting 2), and repair of DNA damage (BRCA and CDKN1A interacting protein). Taken together, a DIGE approach reveals that overexpression of Bcl-x_L not only inhibits apoptosis in the presence of NaBu but also affects cell proliferation and survival in various aspects.

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KEYWORDS: CHO cells; proteomics; Bcl-x_L; sodium butyrate; DIGE analysis; inducible expression

Introduction

Chinese hamster ovary (CHO) cells are widely used for the production of therapeutic proteins including erythropoietin (EPO), antibodies, and antibody-based proteins. In order to meet the increasing demand for these therapeutic proteins, many efforts such as the optimization of culture condition (Clark et al., 2004; Yoon et al., 2004), medium formulation (Castro et al., 1992; Kim et al., 1998), the addition of nutrient supplements (Altamirano et al., 2004; Wong et al., 2005), and genetic engineering to maximize the foreign protein productivity (Borth et al., 2005; Ku et al., 2008) have been performed.

Addition of sodium butyrate (NaBu) has also been used as an efficient method to enhance specific protein productivity (q) in CHO cells (Chang et al., 1999; Kim and Lee, 2000; Laubach et al., 1996; Palermo et al., 1991; Sung and Lee, 2005; Sung et al., 2004). NaBu, a short-chain fatty acid, inhibits the activity of histone deacetylase, and the hyperacetylation of histone proteins induces more access of general transcription factors to DNA (Kruh, 1982; Lee et al., 1993; Riggs et al., 1977). Despite the positive effect of NaBu on q, it can also significantly inhibit cell growth via cell-cycle arrest and cause apoptosis in a dose-dependent manner (Barka, 1998; Calabresse et al., 1993; Chabanas et al., 1985; Kim and Lee, 2000; Mandal et al., 1997; Shao et al., 2004). Thus, the beneficial effect of NaBu on therapeutic protein production can be maximized when apoptotic cell death is reduced by the addition of anti-apoptotic chemicals or the overexpression of apoptosis suppressor genes.

Bcl-x_L, a member of the Bcl-2 family, may operate at two major points in the death pathway: First, Bcl-x_L may regulate the permeability of the outer mitochondrial membrane to cytochrome *c*, which can interact with an apoptotic protease activating factor to form an apoptosome (Kuwana et al., 2002). Second, Bcl-x_L may bind to and inactivate the proapoptotic protein, Bax (Sedlak et al., 1995) and also sequester proapoptotic BH3 proteins, Bim and Bad (Chipuk and Green, 2008; O'Connor et al., 1998; Yang et al., 1995).

There have been many studies to overcome apoptosis and promote cell growth in cell cultures. For example, overexpression of members of the Bcl-2 family including Bcl-2 and Bcl-x_L has been employed to prevent apoptotic cell death induced by various causes such as addition of NaBu

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(Kim and Lee, 2000; Sung and Lee, 2005; Wang et al., 2004), hyperosmolarity (Kim and Lee, 2002), nutrient depletion (Tey et al., 2000), and low culture temperature (Kim and Lee, 2009). However, the intracellular portraits of the CHO cell proteome in response to Bcl-x_L overexpression are still poorly understood despite the number of genetic engineering studies that have been performed.

For the systematic identification and quantification of proteins expressed in a biological system, a gel-based method (e.g., 1D or 2D SDS–PAGE) has been the method of choice for decades and is still widely practiced. This approach is based on statistical analysis using a specific software package that merges and compares several replicate sets of gels for control and treated samples, bearing experimental errors caused from variations in running gels (Righetti et al., 2004). An alternative method is differential in-gel electrophoresis (DIGE). In the DIGE method, control and treated samples are labeled with different colors of dye and separated in a single gel, which minimizes the gel-to-gel variation of spot amount and allows better matching.

In this study, we performed two-dimensional DIGE (2D-DIGE) and MS analysis to investigate changes in the proteome caused by controlled Bcl-x_L overexpression in recombinant CHO (rCHO) cells producing EPO under NaBu treatment. In this approach, we expected to gain insights into the effect of Bcl-x_L overexpression on rCHO cells producing therapeutic protein under a stressful culture condition.

Materials and Methods

Cell Line and Cell Maintenance

The rCHO cell line used in this study was established in our laboratory as described previously (Kim and Lee, 2009). Briefly, the Tet-off system was introduced in EPO-producing rCHO (CHO-EPO) cells and then stable Tet-off CHO-EPO cells were screened by the luciferase assay. The double-stable Tet-off CHO-EPO cells overexpressing hamster Bcl-x_L (EPO-off-Bcl-x_L) were established by co-transfecting a Bcl-x_L expression vector with a pTK-Hyg vector (BD Biosciences Clontech, Palo Alto, CA) into Tet-off CHO-EPO cells, under the control of the Tet system. Tetracycline or doxycycline binds and inactivates tetracycline-controlled transactivator (tTA) which binds Tet response element (TRE) and activates transcription. By using the Tet-off system, the transfected gene is suppressed by the presence of doxycycline and expressed by the removal of doxycycline. They were adapted to grow in suspension with serum-free medium (SFM) (SFM4CHO; HyClone, Logan, UT) supplemented with 250 μg/mL of zeocin (Invitrogen, Carlsbad, CA), 100 μg/mL of hygromycin (BD Biosciences Clontech), 80 nM methotrexate (MTX; Sigma, St. Louis, MO), 4 mM glutamine (HyClone), and 100 ng/mL of doxycycline (BD Biosciences Clontech).

Cell Culture

To determine the effect of Bcl-x_L overexpression in the presence of NaBu, exponentially growing cells in SFM without doxycycline were seeded at 2×10^5 cells/mL into 125 mL Erlenmeyer flasks (Corning, Corning, NY) containing 50 mL of SFM without doxycycline on an orbital shaker (Vision, Incheon, Korea) at 110 rpm in a humidified 5% CO_2 incubator at 37°C. After 3 days of cultivation, NaBu was added to the cultures at a final concentration of 0, 1, 3, and 5 mM, respectively. For a control culture without Bcl-x_L overexpression, exponentially growing cells in SFM with 100 ng/mL doxycycline were used as an inoculum and doxycycline was added to the medium at 100 ng/mL every 3 days. After 3 days of cultivation, NaBu was added to the cultures, as in the cultures with Bcl-x_L overexpression. Cell concentration was estimated using a hemacytometer. Viable cells were distinguished from dead cells using the trypan blue dye exclusion method.

For 2D-DIGE, exponentially growing cells in SFM without doxycycline were inoculated at 2×10^5 cells/mL into two 3-L BIOFLO 110 bioreactors (New Brunswick Scientific, Edison, NJ) with a working volume of 1.8 L. The agitation speed was set at 50 rpm, and the dissolved oxygen concentration was controlled to be equivalent to 50% of the air saturation. The culture temperature and pH were controlled at 37 ± 0.1°C and 7.20 ± 0.02, respectively. After 3 days of cultivation, NaBu was added at a final concentration of 3 mM to the bioreactor. Control cultures were also performed in the bioreactors with exponentially growing cells in SFM with doxycycline.

Cells were harvested 24 h after NaBu addition (on day 4) and washed twice with ice-cold PBS.

Protein Sample Preparation and Protein Content Analysis

To determine cellular protein contents, 1×10^7 cells were lysed for 30 min in 150 μL lysis buffer (30 mM Tris, 7 M urea, 2 M thiourea, 4% CHAPS) and precipitated with 10% TCA/acetone. The precipitate was suspended in the sample buffer (30 mM Tris, 7 M urea, 2 M thiourea, 65 mM dithiothreitol (DTT), 4% CHAPS, and a trace amount of bromophenol blue (BPB)) for protein concentration analysis using the Bradford method.

2D-DIGE and Image Analysis

Bcl-x_L overexpressing cells were labeled with Cy3 and control cells were labeled with Cy5, whereas internal standards were labeled with Cy2 using 400 pM of CyDye (GE Healthcare, Waukesha, WI) for 50 μg protein samples. Labeling was performed for 30 min on ice in the dark. Reactions were then quenched by the addition of 1 μL of lysine (10 mM) for 10 min on ice in dark. Total proteins (150 μg) were mixed and denatured in 2D sample buffer,

and then loaded onto IPG strips (GE Healthcare) after addition of rehydration solution for the first-dimension isoelectric focusing (IEF) at 95,000 Vh. The second dimension was analyzed on 9–17% linear gradient polyacrylamide gels at constant 40 mA per gel until the BPB reached the bottom of the gel. After protein fixation in 40% methanol and 5% phosphoric acid for 1 h, gels were stained with Coomassie brilliant blue G250 for 12 h. Gels were destained with H_2O, scanned in a Bio-Rad GS710 densitometer (Hercules, CA), and then analyzed with the Image Master Platinum 5.0 2-DE image analysis program (GE Healthcare).

The CyDye labeled gels were visualized using a Typhoon 9400 imager (GE Healthcare). Excitation and emission wavelengths were chosen specifically for each of the dyes according to the manufacturer's recommendations using an optimal excitation/emission wavelength for each DIGE fluor: Cy2 (488/520 nm), Cy3 (530/580 nm), and Cy5 (633/670 nm). Images were preprocessed to remove areas extraneous to those of interest using an ImageQuant V2005 (GE Healthcare). Intra-gel analysis was performed using DeCyder difference in-gel analysis (DIA) v6.5.11 and inter-gel matching and statistical analysis were performed using the DeCyder biological variance analysis (BVA) v6.5.11 (GE Healthcare).

In-Gel Tryptic Digestion, MS, and Database Searching

Spots of interest were excised manually from a Coomassie-stained preparative gel and then destained with 50 mM ammonium bicarbonate/40% acetonitrile for 30 min to remove Coomassie blue staining, and dried in a SpeedVac concentrator for 10 min. The protein was digested with 5 μL sequencing grade trypsin (12.5 μg/mL; Promega, Madison, WI) in 50 mM ammonium bicarbonate overnight at 37°C. Trypsinized gel pieces were extracted through a repeated process of hydration–dehydration using 50 mM ammonium bicarbonate and 50% acetonitrile/0.1% formic acid solution. Supernatants were transferred into new tubes, and then dried completely under vacuum.

All mass spectra were acquired in the delayed reflector mode by a Voyager DE-Pro MALDI-TOF/TOF™ Analyzer (Applied Biosystems, Foster City, CA). The proteins were identified by searching the Swiss-Prot and NCBInr databases with the Matrix Science search engine (http://www.matrixscience.com). All mass spectra were searched in the CHO database or in the rodent species database.

Results

An EPO-producing rCHO cell line with inducible overexpression of Bcl-x_L using a doxycycline-regulated gene expression system (Tet-off system) was employed in this study. The doxycycline concentration of 100 ng/mL was high enough to suppress the Bcl-x_L expression to a basal level and did not influence cell growth, EPO production, and cell metabolism such as utilization of glucose and glutamine and production of lactate and ammonia (Kim and Lee, 2009).

Effect of Bcl-x_L Overexpression on Cell Growth Under NaBu Addition

To evaluate the effect of Bcl-x_L overexpression on growth of cells treated with NaBu addition, exponentially growing cells were subjected to different concentrations of NaBu (1, 3, and 5 mM). Cell culture without NaBu addition was also carried out.

Figure 1 shows cell growth and cell viability. When Bcl-x_L was suppressed in the presence of doxycycline, NaBu inhibited cell growth and decreased cell viability in a dose-dependent manner. Maximum viable cell concentration was 4.7×10^6 cells/mL in the absence of NaBu, while it was 2.9×10^6 cells/mL at 1 mM NaBu, 2.3×10^6 cells/mL at 3 mM NaBu, and 2.3×10^6 cells/mL at 5 mM NaBu.

Overexpression of Bcl-x_L did not increase the maximum viable cell concentration. However, it maintained higher viability of cells treated with NaBu. If the culture was terminated at a cell viability of 70%, Bcl-x_L overexpression extended the culture longevity in the presence of NaBu by ca. 3 days.

To assess inducible overexpression of Bcl-x_L, cells were harvested every 3 days for Western blot analysis of Bcl-x_L. Figure 2 shows that Bcl-x_L overexpression was tightly regulated by doxycycline during the culture.

Differential Analysis of Proteomes

To investigate changes between proteomes resulting from Bcl-x_L overexpression in the absence as well as the presence of NaBu, 2D-DIGE was performed using the samples described in the Materials and Methods Section. The samples from 3 mM NaBu addition were selected because Bcl-x_L overexpression significantly improved cell viability in the presence of 3 mM NaBu. For accurate control of culture environmental parameters, such as temperature, pH, and dissolved oxygen concentration, all samples used in the 2D-DIGE were prepared on day 4 from cultures performed in bioreactors.

Figure 3 shows representative 2D-DIGE protein expression maps (PEMs) generated in triplicate. After normalization of the gel images, PEMs of cells with Bcl-x_L overexpression in the absence of doxycycline were then compared to those without Bcl-x_L overexpression in the presence of doxycycline using a BVA module of DeCyder analysis software.

In previous studies where we employed traditional 2D-PAGE and silver staining methods (Baik et al., 2006, 2008), the quantitative comparison of proteomes was performed with spots of an abundant amount because faint spots have greater intra-gel variance due to low reproducibility and variation in the staining step. As a result, most identified proteins were chaperones (ERp 57, GRP 78, and PDI) and

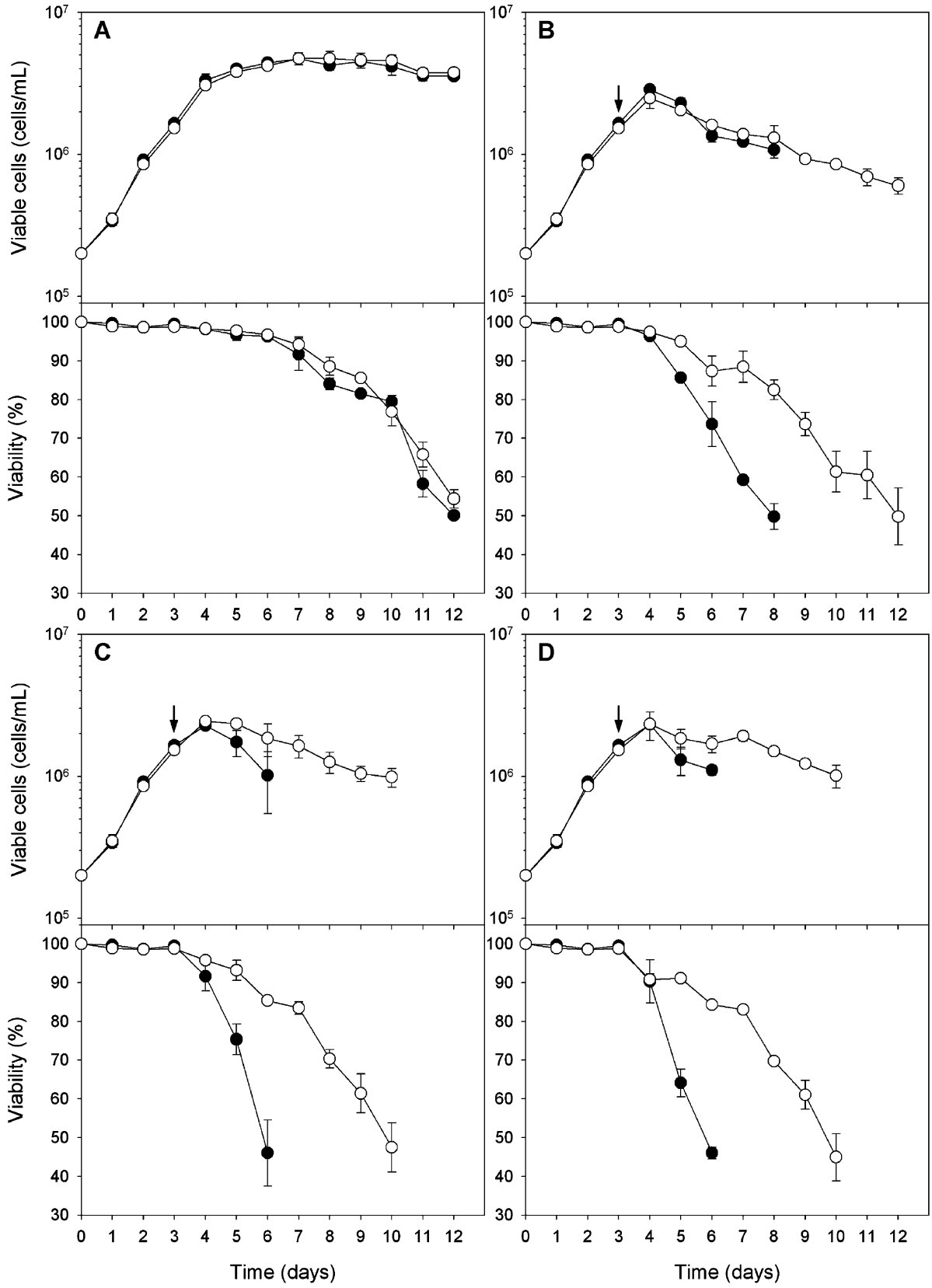

Figure 1. Growth profiles of rCHO cells with Bcl-x_L overexpression (open symbol) and without Bcl-x_L overexpression (closed symbol) in cultures with addition of various concentrations of NaBu. (**A**) 0 mM, (**B**) 1 mM, (**C**) 3 mM, and (**D**) 5 mM. The arrows indicate time of NaBu addition.

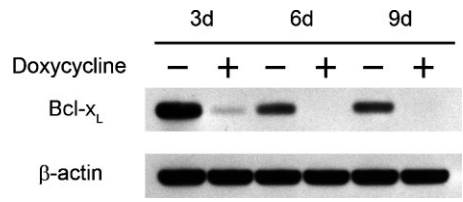

Figure 2. Western blot for controlled expression of Bcl-x_L regulated by doxycycline. The "+" and "–" indicate the presence (100 ng/mL) and the absence of doxycycline, respectively. β-actin was used as a loading control.

HSPs (HSP 70 and HSP 60). Consequently, in this study, the analysis of the differential expression of proteomes was focused on protein spots of small amounts to acquire a range of different proteins regulated by Bcl-x_L overexpression. An increase or decrease of 1.3-fold or more in protein expression with a *t*-test score of ≤ 0.05 was considered as a significant change (or differentially expressed). Ten spots in the cells without NaBu treatment (Fig. 3A) and eight spots in the cells treated with 3 mM NaBu (Fig. 3B) were observed to be differentially expressed by Bcl-x_L overexpression.

Figure 4 shows zoomed images of the differentially expressed spots in Figure 3. Among the 10 differentially expressed spots in the cells without NaBu treatment, 6 spots were up-regulated and 4 spots were down-regulated (Fig. 4A). In the cells treated with 3 mM NaBu, four spots out of the eight differentially expressed spots were up-regulated and four spots were down-regulated (Fig. 4B).

When the differentially expressed spots in Figure 4A and B were compared, three spots (spot no. 1541 in Fig. 4A and spot no. 1266 in Fig. 4B; spot no. 2314 in Fig. 4A and spot no. 1951 in Fig. 4B; spot no. 2320 in Fig. 4A and spot no. 1952 in Fig. 4B) were commonly observed in the same location on gels with a similar regulation ratio, resulting in 15 differentially expressed spots by Bcl-x_L overexpression (Table I).

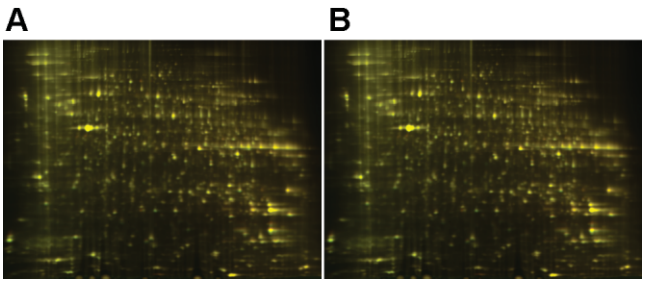

Figure 3. Representative 2D-DIGE protein maps of rCHO cells with Bcl-x_L overexpression and without Bcl-x_L overexpression. (**A**) Cells without NaBu treatment and (**B**) cells treated with 3 mM NaBu. Cells in the bioreactors were harvested on day 4. For treatment of NaBu, 3 mM NaBu was added to the culture on day 3. Cells with Bcl-x_L overexpression were labeled with Cy5 (red), and cells without Bcl-x_L overexpression were labeled with Cy3 (green).

Spot Identification

Peptides retrieved from the differently expressed spots were analyzed by MALDI-TOF–MS. Since the complete database of CHO cells has not been established yet, all mass spectra were also searched in the database of rodent species or all entries.

Figure 5 shows the representative MALDI mass spectra of spot no. 1266 (also spot no. 1541 in the control gel without NaBu addition) and spot no. 1952 (also spot no. 2320 in the control gel without NaBu addition). These spots were identified as Iq motif-containing GTPase-activating protein 1 (IQGAP1) and ferritin heavy chain (FHC), respectively. Out of the 15 protein spots, 13 spots were successfully identified by MALDI-TOF–MS. Spot no. 2848 in the control gel without NaBu addition could not be identified due to a low amount of protein peak acquisition. Spot no. 2082 from the gel of 3 mM NaBu addition was not matched with a protein database, resulting in a value of $P > 0.05$. The identified proteins were categorized according to their biological function in Table I.

Discussion

NaBu has been used to increase the expression of foreign proteins in rCHO cell culture (Chang et al., 1999; Kim and Lee, 2000). NaBu is also well known as an anti-cancer drug due to its exertion of apoptotic effects on various types of cancer cells (Canes et al., 2005; Sonnemann et al., 2008; Zhu and Otterson, 2003). A recent genomic and proteomic study suggested that NaBu affects protein processing and vesicle trafficking genes (Yee et al., 2008).

Since dead cells negatively affect the quality of protein product and product yield (Arden and Betenbaugh, 2004), there have been many efforts to maintain high cell viability and culture longevity by overexpressing anti-apoptotic genes (Figueroa et al., 2004; Fussenegger et al., 1998; Hwang and Lee, 2009). In this study, Bcl-x_L was overexpressed using the Tet-off system, doxycycline-controlled inducible expression system to inhibit apoptotic cell death induced by the addition of NaBu. Clonal variation may give rise to unexpected experimental results because even rCHO cell lines originating from the same transfected cells show different characteristics such as cell growth and specific productivity according to changes of culture conditions (Yoon et al., 2004). Clones from the same transfected cells are different cell lines in the strict sense. Using this type of controlled expression system leads to make a comparison in the same cell line, not in different clones which can exclude the possibility of clonal variability that is usually encountered in a constitutive overexpression system.

A proteome analysis using 2D-DIGE and MS analysis revealed that Bcl-x_L overexpression altered the pattern of protein expression of rCHO cells in the presence as well as in the absence of NaBu. As summarized in Table I, three

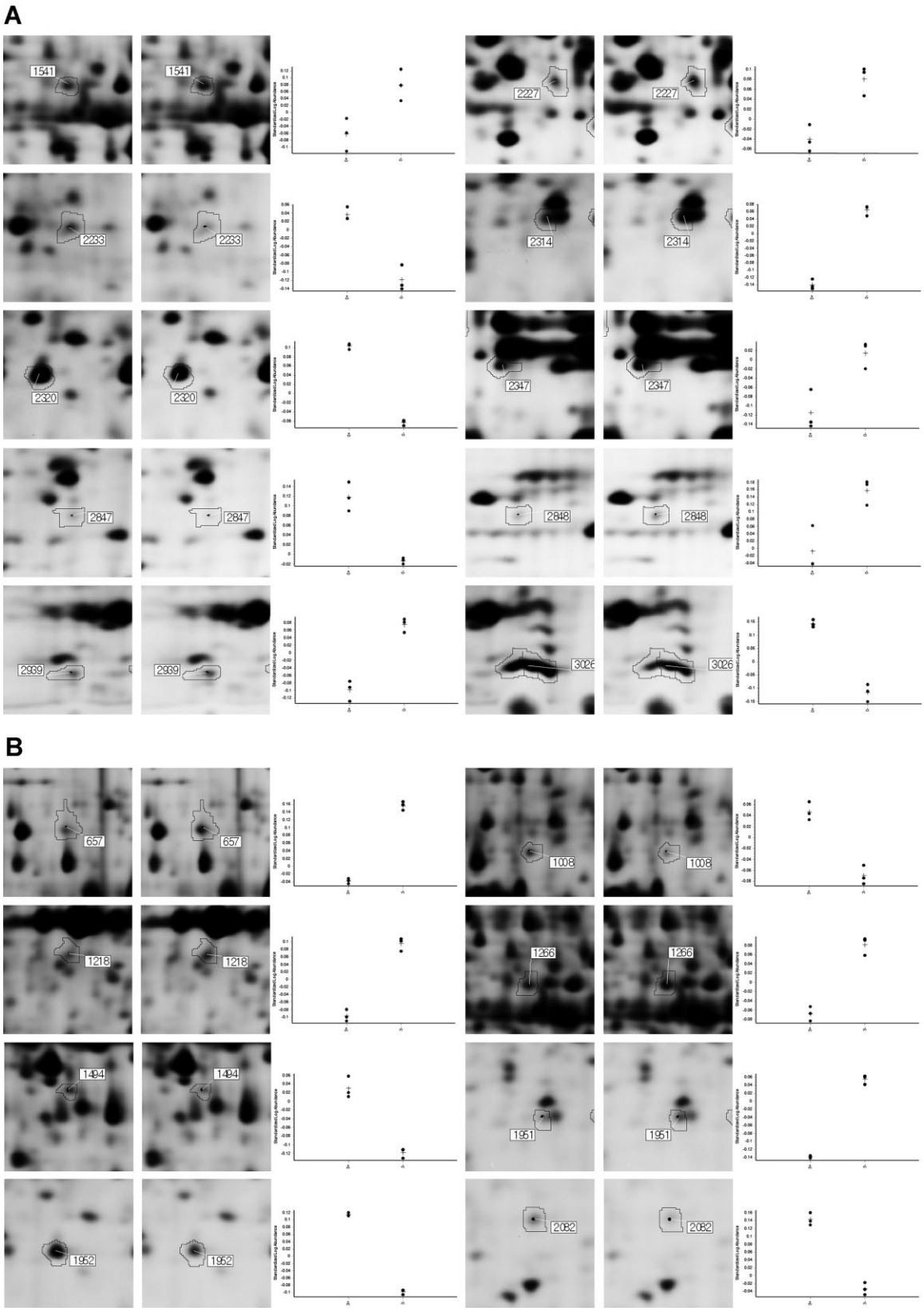

Figure 4. Zoomed images of the differentially expressed spots. (**A**) Zoomed image from Figure 3A and (**B**) zoomed image from Figure 3B. Left columns indicate the gel images of no Bcl-x_L overexpressing cells and right columns indicate the gel images of Bcl-x_L overexpressing cells. Dots indicate relative spot intensities in triplicate. Ratios of expression levels are represented in Table I.

Table I. Identification of Regulated Protein Spots by MALDI-TOF–MS

Spot no.[a]	Score[b]	% coverage[c]	Accession no.[b]	Name	Average ratio[d]	*t*-test[d]	Biological process
657 (NB3)	101	29	gi|78365255	Dihydrolipoamide-*S*-acetyltransferase (DALT)	1.57	1.3E − 5	Glycolysis and biosynthesis
1008 (NB3)	74	23	gi|95113671	Mitochondrial processing peptidase beta subunit (MPPB)	−1.31	1.1E − 3	Mitochondrial protein maturation
1218 (NB3)	86	37	gi|41054806	Guanine nucleotide binding protein (G protein), alpha inhibiting 2 (GNAI2)	1.55	1.3E − 4	Signal transduction
1494 (NB3)	128	42	gi|1174694	Thiosulfate sulfurtransferase (TST, rhodanese)	−1.41	7.3E − 4	Cyanide detoxification
1541 (NB0)	81	23	gi|27370648	Iq motif-containing GTPase-activating protein 1 (IQGAP1)	1.38	2.0E − 2	Cell adhesion and migration
1266 (NB3)					1.41	5.0E − 4	
2314 (NB0)	69	49	gi|148685831	BRCA2 and CDKN1A interacting protein, isoform CRA_d (BCCIP, TOK1)	1.60	5.8E − 5	DNA damage repair
1951 (NB3)					1.56	1.0E − 5	
2320 (NB0)	108	44	gi|120515	Ferritin heavy chain (FHC)	−1.47	5.8E − 6	Ion storage
1952 (NB3)					−1.63	1.5E − 6	
2227 (NB0)	77	43	gi|1170100	Glutathione-*S*-transferase P (GST class-pi)	1.32	6.3E − 3	Detoxification
2233 (NB0)	71	22	gi|8393296	Eukaryotic translation elongation factor 2 (EF2)	−1.43	1.5E − 3	Protein translation
2347 (NB0)	71	10	gi|182637561	Axonemal heavy chain dynein type 3 (DNAH3)	1.35	1.3E − 2	Intracellular molecule transfer
2847 (NB0)	92	60	gi|223582	Histone H4	−1.36	1.7E − 3	Nucleosome formation
2939 (NB0)	84	24	gi|73974625	Similar to potassium voltage-gated channel KQT-like protein 3 (KCNQ3)	1.49	7.8E − 4	Potassium transportation
3026 (NB0)	81	64	gi|6755392	S100 calcium-binding protein A6 (S100A6, calcyclin)	−1.81	2.3E − 4	Ubiquitination and degradation of β-catenin

[a]Spot numbers were from the 2D gels shown in Figure 4A and B.
[b]Protein accession numbers and MS protein scores were obtained by searching the Swiss-Prot and NCBInr databases with the Matrix Science search engine (http://www.matrixscience.com).
[c]Amino acid sequence coverage for the identified proteins by MS fingerprinting.
[d]Average ratio Bcl-x_L overexpressing/no Bcl-x_L overexpressing and *t*-test value were quantified by DeCyder BVA module.

proteins were commonly regulated with a similar ratio of expression level by Bcl-x_L overexpression. Regardless of NaBu treatment, Bcl-x_L overexpression increased the expression level of IQGAP1 and BRCA2 and CDKN1A (p21, Cip1, and Waf1) interacting protein (BCCIP) isoform CRA_d, while decreased that of FHC.

IQGAP1 is a multi-domain protein that binds to a wide variety of targets and modulates several cellular activities, including cell–cell adhesion, transcription, cytoskeletal architecture, and signaling pathways. Increased expression of IQGAP1 was frequently observed in various cancer cell lines (Johnson et al., 2009). In addition, IQGAP1 expression was significantly decreased when apoptosis was induced by anti-tumor reagents in a human hepatoma cell line (Zhou et al., 2009), while overexpression of IQGAP1 enhanced proliferation of epithelial cells (Jadeski et al., 2008), suggesting that up-regulation of IQGAP1 is related to cell survival enhanced by Bcl-x_L overexpression. BCCIP, also named Tok-1, plays a critical role in resolving spontaneous DNA damage (Lu et al., 2007). Up-regulation of BCCIP by Bcl-x_L overexpression may also contribute to cell survival by reducing DNA damage.

FHC, one of the two subunits of ferritin, is a highly conserved and ubiquitously expressed major intracellular iron storage protein (Torti et al., 1988). Unlike the ferritin light chain (FLC), FHC, which has ferroxidase activity involved in ion sequestration, is induced by tumor necrosis factor (TNF) in a nuclear factor-κB (NF-κB)-dependent manner (Pham et al., 2004). Since Bcl-x_L inhibits TNF-induced apoptosis (Gozzelino et al., 2008; Srinivasan et al., 1998), it is not surprising that expression of FHC was reduced by Bcl-x_L overexpression.

Besides the commonly regulated proteins mentioned above, dihydrolipoamide-*S*-acetyltransferase (DLAT) and guanine nucleotide binding protein, alpha inhibiting 2 (GNAI2) were differentially up-regulated, whereas thiosulfate sulfurtransferase (TST, rhodanese) and mitochondrial processing peptidase beta subunit (MPPB) were down-regulated in response to Bcl-x_L overexpression in the presence of 3 mM NaBu.

DLAT is the E2 component of pyruvate dehydrogenase complex, a ubiquitous mitochondrial matrix protein associated with the inner mitochondrial membrane. DLAT catalyzes oxidative decarboxylation of pyruvate, which is the link between glycolysis and the citric acid cycle (Berg et al., 2002). The up-regulation of these enzymes involved in the metabolic pathway for glycolysis and biosynthesis reflects a metabolic shift by the enhanced viability and proliferation rate resulting from Bcl-x_L overexpression at 3 mM NaBu.

GNAI2 is one of the three proteins that inhibit the human G protein α subunit, one of the three members composing the G protein, which acts as a signal transducer linking receptors and effectors (Itoh et al., 1988). Overexpression of GNAI2 enhanced proliferation of mouse fibroblast cells, while inactive mutation of GNAI2 impaired cell growth (Hermouet et al., 1991). Bcl-x_L overexpression did not affect GNAI2 expression in the absence of NaBu. This result

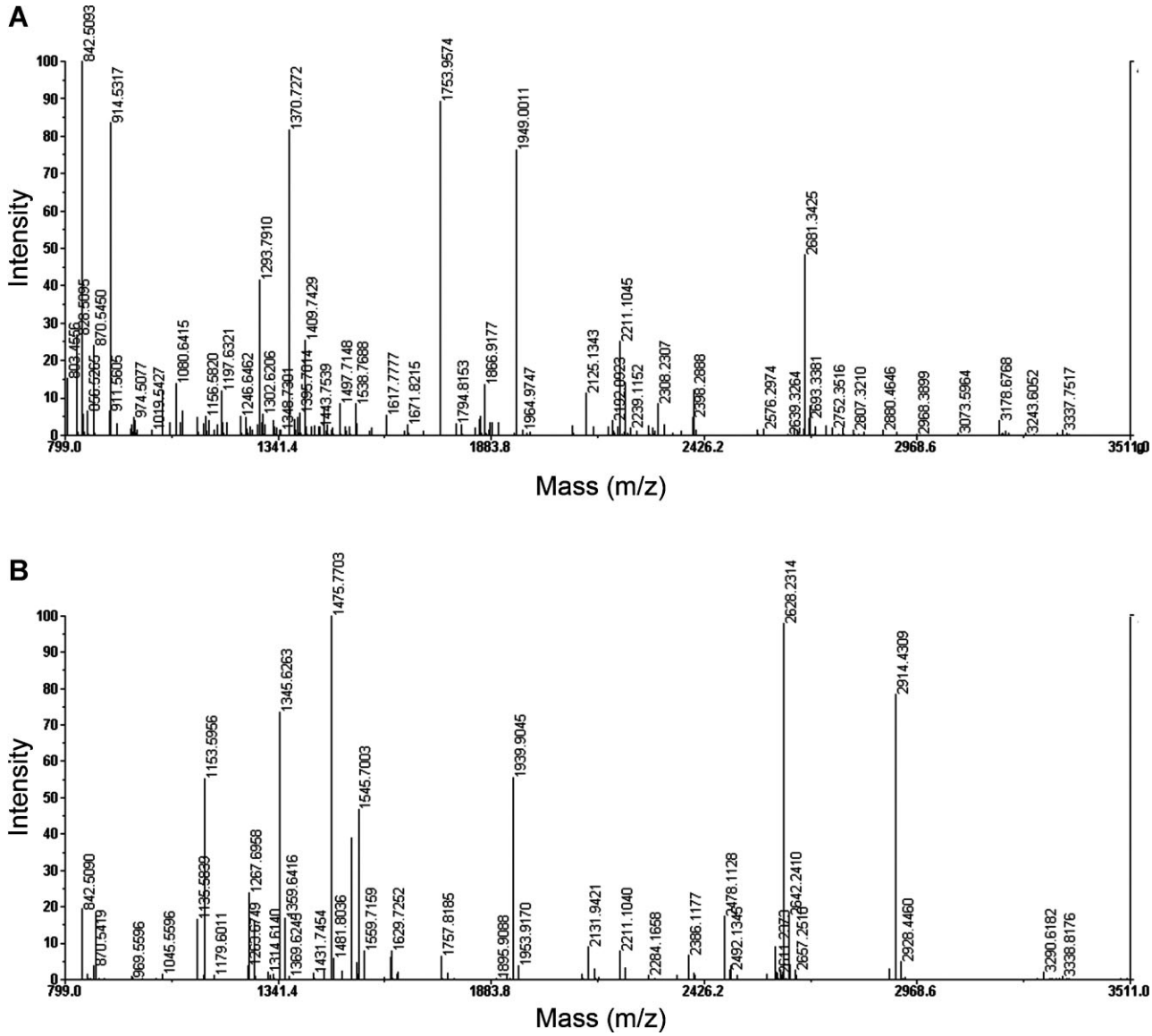

Figure 5. MALDI-TOF mass spectrum of spot no. 1266 which was identified as Iq motif-containing GTPase-activating protein 1 (**A**) and spot no. 1952 which was identified as ferritin heavy chain (**B**).

implies that the reduced expression level of GNAI2 by NaBu addition, which hindered cell growth, might be restored by Bcl-x_L overexpression.

TST, also known as rhodanese, is a mitochondrial matrix enzyme that has sulfurtransferase activity, transferring the sulfur atom from thiosulfate to cyanide to produce thiocyanate (Westley and Rochefort, 1980). In a genomic study using microarrays, the TST gene expression in colonic mucosa from cancerous tissues with a high cell growth rate was significantly decreased, compared with that from normal tissues (Birkenkamp-Demtroder et al., 2002). Furthermore, the TST activity and expression were significantly increased by butyrate and histone deacetylase inhibitors, which decrease cell growth rate (Ramasamy et al., 2006). These reports are in accordance with our observation that the expression level of TST was increased in the condition of NaBu addition without Bcl-x_L expression (reduced cell growth), and then decreased by Bcl-x_L overexpression (enhanced cell proliferation).

MPPB is a β subunit of mitochondrial processing protein (MPP) which, in concert with a mitochondrial intermediate peptidase, cleaves most mitochondrial proteins encoded by nuclear genes and imported into the mitochondria (Paces et al., 1993). In the mitochondrial pathway of apoptosis, or type I apoptosis, BH3-only proteins may translocate into mitochondria to activate Bax/Bak (Paschen et al., 2007), suggesting that expression of MPP is probably affected by the onset of apoptosis.

In conclusion, Bcl-x_L overexpression in CHO cells treated with 3 mM NaBu resulted in the differential expression of various proteins besides the proteins involved in the anti-apoptotic pathway which are known to be regulated by Bcl-x_L. These proteins were involved in cell survival, cell proliferation, and repair of DNA damage, suggesting that the consequences of Bcl-x_L overexpression affect cell growth in various aspects other than apoptosis inhibition.

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