# *Proteomic analysis of the adaptation of the host NS0 myeloma cell line to a protein-free medium*

RESEARCH

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***ABSTRACT***

The NS0 myeloma cell line is often used for the production of monoclonal antibodies (Mabs) and other recombinant proteins. The growth of these mammalian cells in a protein-free media has several advantages including cost, safety, consistency, efficiency and regulatory approval. However, the adaptation of the NS0 myeloma cell line so as to grow in a protein-free medium is poorly understood. In order to better understand this process, we applied proteomic techniques, specifically two-dimensional electrophoresis (2DE) and mass spectrometry (MS), to identify the key pathways involved in this adaptation. The analysis of changes in protein expression between the host myeloma cell line and two recombinant NS0 cell lines expressing different humanized Mabs has primarily revealed changes in proteins associated with carbohydrate metabolism, energy production, protein synthesis and folding, membrane transport and cell proliferation. Other factors that may be involved in the adaptation of NS0 myeloma cells to a protein-free medium are reported.

*Keywords*: mass spectrometry, myeloma cell line, protein-free medium, two-dimensional electrophoresis

*Biotecnología Aplicada 2008;24:215-223*

***RESUMEN***

**Análisis proteómico de la adaptación de los receptores de línea celular de mieloma NS0 en medios libres de proteínas.** La línea celular de mieloma NS0 es comúnmente usada para la producción de anticuerpos monoclonales y otras proteínas recombinantes. El crecimiento de estas células en medios libres de proteínas presenta varias ventajas entre las que se encuentran el costo, la seguridad, la eficiencia y la aprobación de compañías reguladoras. No obstante, la adaptación de esta línea a crecer en medio libre de proteínas no es completamente conocida. Con el objetivo de ayudar en la comprensión de este proceso, nosotros hemos aplicado técnicas proteomicas, específicamente electroforesis bidimensional (2DE) y espectrometría de masas (MS), para identificar proteínas esenciales involucradas en la adaptación. Análisis de cambios en la expresión de proteínas entre la línea hospedera y dos líneas recombinantes productoras de anticuerpos monoclonales han revelado cambios en proteínas asociados al metabolismo de los carbohidratos, producción de energía, síntesis y renaturalización de proteínas, transporte de membranas y proliferación celular. También son reportados otros factores que pudieran estar involucrados en la adaptación de la línea celular NS0 a medio libre de proteínas.

*Palabras clave*: espectrometría de masas, línea celular de mieloma, medios libres de proteínas, electroforesis bidimensional

## *I*ntroduction

Mammalian cells are often used to manufacture recombinant proteins for therapeutic, diagnostic and research use. After host cell transfection and clonal selection, cells are adapted to grow in the culture environment used in protein production. The inclusion of animal serum (typically bovine) in a growth medium has many disadvantages such as high cost and the introduction of extraneous factors in the culture, which must be removed during protein purification. Adaptation generally involves passing cells over a relatively long period of time so that they gradually develop the ability to grow in a new environment [1-3]. Much effort has therefore been devoted to developing serum- and protein-free media for the production of recombinant proteins.

Serum is a complex mixture whose components, including growth factors, plasma proteins, metabolites and hormones, play a crucial role during normal cell culture. Serum often provides the nutrients necessary for cell growth, which either may not be present in the basal medium or are present in insufficient amounts to sustain cell growth at an exponential proteins may stabilize and modulate the action of the substances which they bind, or in some cases may detoxify the medium by binding toxic metals and pyrogens. Serum is a source of factors that may be necessary for the proper attachment and spreading of cells on the plastic culture substrate. Serum also buffers the pH of the growth medium and protects the cells from damage due to excreted proteases. When serum is omitted from the culture medium, substitutes must be found for all of these critical functions [4].

The NS0 murine myeloma cell line has become one of the most popular systems for large-scale heterolo-

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gous protein expression, especially for the production of recombinant Mabs. The popularity of this cell line is in part due to its capacity to stably and productively incorporate foreign DNA, the lack of endogeneous antibody production, and its extensive regulatory pedigree, being a subclass of the NS-1 cell line [5]. NS0 cells grow as single-cell dispersions when cultured in suspension, and exhibit robust growth and high levels of protein production in a variety of selection and production environments. They are one of the murine myeloma cell lines available for the construction of hybridomas and transfectomas [6].

However, the adaptation mechanism of the NS0 myeloma cell line to the protein-free medium is not well understood. NS0 cells have demonstrated a particular susceptibility to apoptosis when cultured under low serum conditions. A number of factors have been postulated to contribute to this, including a lack of HSP 70 expression and the variability in membrane cholesterol concentration that can be induced by different culture conditions [7]. It has been observed that the maintenance of environmental cholesterol levels by the exogenous supplementation of the growth media can reduce apoptosis in suspension cultures [8]. As the requirement for growth of mammalian cells in the serum-free medium developed for exogenous protein expression, it was discovered that the NS-1 myeloma cell line and its derivatives (including NS0) are auxotrophs for cholesterol [9, 10]. Changes in cyclin A, cyclin B and transcription factor E2F-1 expression levels have already been reported during the adaptation of Chinese hamster ovary (CHO) cells to a protein-free medium [11].

Protein expression analysis is performed by studying the proteome, 2DE and the various ways these proteins are detected, and subsequently, identified have been described in detail [12-17].

In this study, we used 2DE and MS analysis to characterize changes in protein expression between the host NS0 cell line and transfected cell lines expressing two different Mabs, before and after their adaptation to growth in a protein-free medium. Total protein fractions were used during the study in order to find the main cellular pathways related to this process. Possible adaptation mechanisms and their relation to cell growth are discussed.

## Materials and methods

### Materials

IPG strips, carrier ampholytes, urea, thiourea, CHAPS, DTT, acrylamide solution, SDS and glycerol were purchased from BioRad labs (UK). All reagents used for staining and sample preparation were from SPECTRUM (USA) and Riedel de Haen (Germany). ASB-14 was supplied by Calbiochem (UK). Anti-dynein LC, anti-prohibitin and goat anti-rabbit IgG-HRP antibodies were supplied by Santa Cruz Biotechnology (USA).

### Cell line and culture

Three cell lines were used in this study:

NS0: murine NS0 myeloma host cell lines adapted and non-adapted to a protein-free medium.

NS0/hR3: NS0 cell line expressing anti-human EGF receptor Mab adapted and non-adapted to a protein-free medium [18, 19].

NS0/hT1hT: NS0 cell line expressing anti-human CD6 Mab adapted and non-adapted to a protein-free medium [20].

Cell lines were obtained from the cell banks of the Centre of Molecular Immunology (Cuba); and adapted as previously reported [21]. The Mab-producing clones were obtained by the limiting dilution cloning method [22]. Expression stability studies were carried out in 25 $cm^2$ T-flasks in duplicate using cells that were sub-cultured every 3 or 4 days over 45-50 days. After 10 days the concentration of IgG in the supernatant was tested using an anti-human IgG sandwich ELISA [23].

Kinetic studies were carried out in triplicate in 250 mL spinner flasks (Integra, Switzerland). Samples were taken daily or twice per day for cell counting by the trypan blue exclusion method. After that, samples were centrifuged and stored at -70 °C for IgG concentration measurement.

Cells were cultured in 75 $cm^2$ flasks in PFHM-II (protein-free medium) (GIBCO BRL, USA) supplemented with 1% (v/v) foetal bovine serum (FBS) (GIBCO BRL, USA) or in PFHM II. Cells were maintained in an atmosphere of 5% $CO_2$, 95% humidity, at 37 °C and were passed 1:5 into a fresh medium every 2 to 3 days. Growth parameters from these cell cultures in the presence or absence of serum are shown in the table 1. When the cultures were in the exponential growth phase, cells obtained from both culture conditions were frozen in a medium supplemented with 5% FBS and DMSO (20 x $10^6$ cell/vial) in order to maintain cell characteristics and viability. Vials were stored at -70 °C.

### Metabolite concentration

Glucose, lactate and cholesterol concentrations were determined by kits supplied by Centis Diagnósticos (Cuba).

### *Sample preparation*

Cells from NS0, NS0/hR3 and NS0/hT1hT cell lines, grown in PFHM II supplemented with 1% FBS ("non adapted" condition) and grown in a protein free medium ("adapted" condition) (corresponding to 20 x $10^6$ cells each) were thawed slowly and washed three ti-

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Table 1. Cellular growth parameters from the host NS0, NS0/hR3 and NS0/hT1hT cell lines adapted and non-adapted to a protein-free medium (PFM). $Xv_{max}$: maximum viable cell concentration, $Xt_{max}$: maximum total cell concentration, $\mu_{max}$: maximum growth rate, $IgG_{max}$: maximum IgG production, $q_{IgG}$: specific IgG production rate

| Cell line | $Xv_{max}$ (cell x $mL^{-1}$) | $Xt_{max}$ (cell x $mL^{-1}$) | $\mu_{max}$ cell x $mL^{-1}$ x $h^{-1}$) | $IgG_{max}$ ($\mu$g x mL) | $q_{IgG}$ ($\mu$g x $mL^{-1}$ x $h^{-1}$) |
|---|---|---|---|---|---|
| Host NS0 (FBS) | $2.14e^{+06}$ | $2.79e^{+06}$ | 0.034 | - | - |
| Host NS0 (PFM) | $1.55e^{+06}$ | $2.23e^{+06}$ | 0.036 | - | - |
| NS0/hR3 (FBS) | $1.26e^{+06}$ | $1.38e^{+06}$ | 0.025 | 10.70 | $2.31e^{-07}$ |
| NS0/hR3 (PFM) | $1.51e^{+06}$ | $1.58e^{+06}$ | 0.037 | 41.50 | $4.73e^{-07}$ |
| NS0/hT1hT (FBS) | $2.34e^{+06}$ | $2.45e^{+06}$ | 0.028 | 46.50 | $1.44e^{-07}$ |
| NS0/hT1hT (PFM) | $1.15e^{+06}$ | $1.49e^{+06}$ | 0.035 | 25.90 | $1.33e^{-07}$ |

mes with phosphate-buffered saline (PBS) (140 mM NaCl, 2.6 mM KCl, 8.1 mM $Na_2PO_4$, 1.4 mM $NaH_2PO_4$, pH 7.4). After the last wash, the supernatants were carefully removed and the cell pellets lysed by adding lysis-rehydration buffer (7M urea, 2M thiourea, 1% (w/v) DTT, 0.5% (w/v) ASB-14, 2% (w/v) CHAPS, 0.5% (v/v) ampholytes 3-10), for 30 min at room temperature. Samples were centrifuged (15 min at 13000 rpm, room temperature) the supernatant was collected and incubated with RNAse (2mg/mL) at 4 °C for 10 min. Samples were then ultracentrifuged for 3h at 60000 x g, at 20 °C. Proteins in the resultanting supernatant were then precipitated by adding cold acetone containing 5% (v/v) trichloroacetic acid (TCA) in a proportion of 1:5 sample: acetone solution and incubated for 1h at -20 °C. The pellet was washed five times with cold acetone (100%) and centrifuged at 13000 rpm, 15 min at room temperature. Finally, the pellets were solubilized in lysis-rehydratation bu-ffer for 30 min, before adding of 2.5% (w/v) acrylamide for 30 min. Total protein was determined using the Bradford assay (BioRad, USA) [24].

## SDS-PAGE and Western blot

Samples were dissolved in 4X SDS-sample buffer and briefly spun in a microfuge to remove insoluble particles before loading on a 15% polyacrylamide SDS-gel (2-5 µg per lane).

Gels were run, semi-dry-blotted onto a PVDF-membrane as described in the manual (Immobilon P, Millipore, USA), then blocked overnight in 5% non-fat dry milk in PBST (0.5% Tween-20 in PBS), incubated for 1h at 37 °C with the primary antibody (anti-dynein LC or anti-prohibitin, diluted 1:500) in the blocking buffer, washed for 15 min with 3 changes of PBST, incubated for 1 h at 37 °C with the secondary antibody (goat anti-rabbit IgG-HRP, diluted 1:500) in the blocking buffer and washed for 15 min with 3 changes of PBST. Membranes were incubated for 5 min in a peroxidase substrate (Sigma, USA) prior to protein detection.

### *Two-dimensional electrophoresis*

For each condition, three IPG strip replicates were focused in parallel, (3 strips per cell line in each condition, with a total of 18 strips). The strips (pI range 3 to 10) were rehydrated passively overnight in 350 µL lysis buffer containing 200 µg total protein extract, followed by isoelectric focusing (Protean IEF cell, BioRad) at 250 V for 15 min, ramping to 4000 V for 6 h, and focused for a total of 75000 Vh. The IPG strips were then equilibrated in buffer (6M urea, 2% (w/v) SDS, 15% (w/v) glycerol, 0.375 M Tris-HCl, pH 8.8) for 20 min, before separating in the second dimension. After equilibration, the IPG strips were placed onto 15% polyacrylamide gels and electro-phoresed at 16 mA/gel, 15 °C for 30 min followed by 32 mA/gel for 4h. Proteins were visualised by silver staining [25]. Timing during silver staining was strictly controlled to improve reproducibility in image development.

### *Image analysis*

The stained gels were scanned using an Image Master scanner (GE Healthcare, USA) and the images were exported to the image analysis software program Melanie 5 (Genebio, Switzerland). For each gel (three gels per condition), the spots were detected and quantified automatically using default spot detection parameters from the software program. Spots were quantified in terms of their relative volume (% vol), which is the volume of an individual spot, related to the sum of the volumes of all spots in the image and then multiplied by 100. The % vol was calculated in order to correct for differences in protein loading and gel staining between different gels. Reported data on spot quantification corresponds to averaged values for the three replicate gels for each condition. Gel spots were assessed by their spot positions using the 2D image from the host NS0 cell line cultured in the medium supplemented with 1% FBS as the reference gel. Manual editing was performed for each spot group to assure matching quality.

To evaluate quantitative changes in protein expression from differentially treated cells, the analysis of 2D gels was performed using multifactorial correspondence analysis and Student's t-test implemented in Melanie 5 software. Correspondence analysis can be used to summarise complicated datasets that involve many variable, into a 2D factorial space. Consequently, it is possible to determine the similarity of different gels and characteristic spots in these gels. For spots to be considered for statistical analysis, a criterion was set for their presence in all three gels from two independent experimental sets; % vol values of these spots were then determined. ANOVA and Kruskall Wallis tests for spot abundance analysis and euristic clustering analysis for gel reproducibility were also employed. Only spots with ≥2 fold change were chosen for further analysis.

### *Preparative 2DE*

The same conditions of analytical 2DE were performed. Only the sample concentration was increased in five fold. Proteins were visualised by silver staining compatible with MS analysis [25].

### *In-gel digestion*

Spots were excised from preparative gels (proteins corresponding to 100 x $10^6$ cells/gel). After excision, the spots of interest were destained and digested as detailed previously [26].

## Matrix-Assisted Laser Desorption/ Ionization mass spectrometry (MALDI-MS)

Following gel digestion, samples were resuspended in 10 µL of 0.1% (v/v) formic acid, desalted using C-18 Zip Tips (Millipore, USA) and 0.5 µL of the sample was spotted onto a MALDI target followed immediately by 0.5 µL of α-cyano-4-hydroxy-transcinnamic acid in 0.1% (v/v) TFA/60% (v/v) ACN. MALDI mass spectrometry was performed on a Vo - yager DE-STR TOF mass spectrometer (Applied Biosystems, USA), operated in a reflectron mode, with delayed extraction. Spectra were obtained by accumulating 100 consecutive laser shots. Calibration was performed using $[M+H]^+$ ions of a mixture of RFDS (m/z 524.24), bradykin (m/z 904.47), angiotensin II (m/z 1046.54), angiotensin III (m/z 1296.68), neurotensin (m/z 1672.92), KRELVEP (m/z 2318.28) and insulin β chain (m/z 3495.00).

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### Liquid chromatography electrospray ionisation tandem mass spectrometry (LC-ESI-MS-MS)

Peptide identification by LC-ESI-MS-MS was performed using a QTof1 (Micromass, UK), coupled online to an Ultimate/Switches/Famos nanoflow LC system (Dionex, UK). Typical gradients were 0-60% B (95% (v/v) ACN, 0.05% (v/v) formic acid) (buffer A: 2% v/v MeCN, 0.05% v/v formic acid) over 55 min, at a flow rate of 180 nL/min. Automatic function switching was performed on the column eluate, with up to 3 switching events per MS survey scan. ProteinLynx PeptideAuto module in MassLynx 3.4 was used to generate .pkl files for the interrogation of the rodent SwissProt database via an in-house MASCOT Server (Matrix Science, UK).

### Protein identification using database searching algorithms

Both MALDI-MS data and data acquired following LC-ESI-MS-MS were searched against rodent SwissProt database using the MASCOT algorithm [27]. Search parameters including modifications, such as cysteine propionamidation and methionine oxidation were considered. The tolerance in the precursor ion m/z was ± 200 ppm, and the tolerance in the product ion m/z was ± 200 mTh. Two missed cleavage sites were allowed in the protein identification. Mascot score of ≥29 was considered significant for positive identification of a peptide.

The LC-ESI-MS-MS spectra were manually inspected and considered as reliable identifications when four or more consecutive C-terminal $y_n$ ions were assigned to intense signals (signal/noise ≥3), usually substantiated by the presence of one or more b ions. The identity of each protein from a characterized peptide was checked by FASTA searching the EMBL-EBI database. Analysis of subcellular location and molecular function of proteins of interest was performed using SwissProt, KEGG and ¡HOP databases [28-33].

## Results

### Adaptation of the host NS0 myeloma cell line to the protein-free medium

To characterize the changes associated with the adaptation to the protein-free medium 2DE gels of protein extracts from the cell line cultured in PFHM II with or without 1% (v/v) FBS were compared over a pI range of 3 to 10 (Figure 1). The PFHM II medium is a commercially available protein-free hybridoma medium from GIBCO. The chemical ingredients and concentrations of this medium are not publicly available. Previous results have demonstrated that this medium is supplemented with amino acids and salts to substitute some protein functions, for example; ferric citrate acts as an iron carrier, a function usually performed by transferrin in FBS [34].

During sample preparation, experimental conditions were controlled to maintain reproducibility. In particular, the effect of freezing and thawing on the 2DE protein profile was evaluated. For this, samples obtained following different freezing protocols and different times of thawing were compared with fresh sample processed immediately after collection. No significant differences were found in the protein profile (results are shown as supplementary material in Table 1 in http://www.cim.sld.cu/publicaciones/2006/tables[1]%20final.pdf). In addition, differences in samples collected in G1, S, M, and G2 phases of the cell cycle in non-adapted and adapted cell lines were not found (supplementary material, Table 2 in http://www.cimsld.cu/publicaciones/2006/tables[1]%20final.pdf).

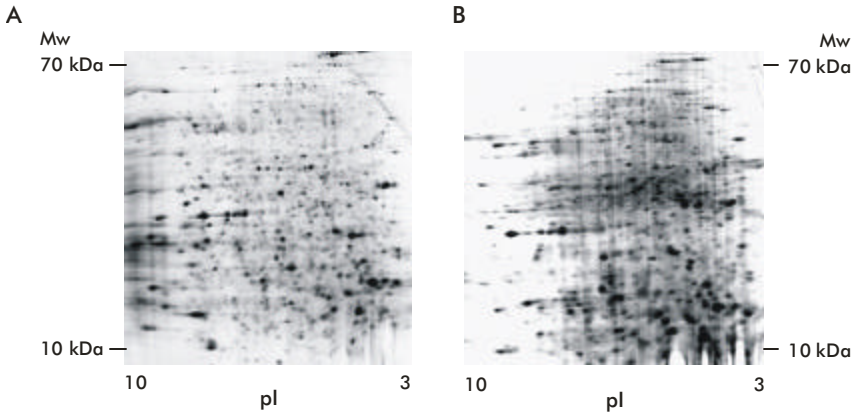


Figure 1. Silver-stained 2D gels corresponding to the host NS0 myeloma cell line adapted (A) and non-adapted (B) to a protein-free medium. These are representative images from three replicate gels.

Following adaptation to the protein-free medium, 78 spots changed their intensity by a factor of ≥2 from 1200 detected spots/ treatment. Interestingly, the majority of differentially expressed proteins decreased their expression in cells adapted to the protein-free medium. Fifty eight proteins were characterized by MALDI-MS and/or LC-ESI-MS-MS (Figure 2 and Table 2). Peptide sequences are shown in supplementary table 3 (http://www.cim.sld.cu/publicaciones/2006/tables[1]%20final.pdf).

Proteins involved in carbohydrate metabolism, such as pyruvate kinase (- 9.8 fold) and alpha enolase (-6.1 fold) and transport through the cytoplasmic membrane, such as the potassium channel subfamily K member 15 (- 8.0 fold), were down-regulated after adaptation to the protein-free medium. In contrast, proteins related to protein synthesis and cytoskeleton structure such as the elongation factor 1 alpha 1 (+ 8.8 fold), eukaryotic initiation factor 4A-II (+ 8.7 fold), poly (rC)-binding protein (+ 9.2 fold),

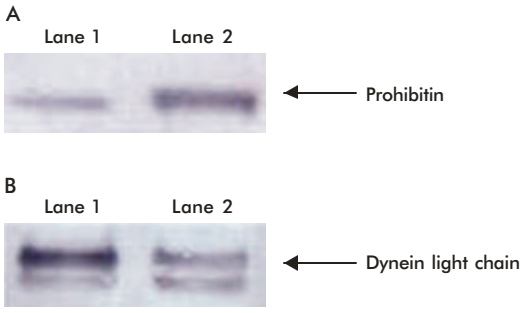


Figure 2. Western blotting of NS0 cell lines with antibodies against either (A) prohibitin or (B) dynein light chain either after (lane 1) or before (lane 2) adaptation to a protein-free medium.

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Table 2. Fold change in protein expression levels following adaptation of the host NS0 cell line to a protein-free medium. Protein analysis was performed by MALDI-MS or LC-ESI-MS-MS following in-gel digestion of 2D silver stained spots

| Protein name | Theorical pI | Theorical Mw (kDa) | Accession number (SwissProt) | % of sequence coverage (MASCOT score) by MALDI-MS | Peptide number (MASCOT score) by LC-ESI-MS-MS | Fold change (adapted/non-adapted) |
|---|---|---|---|---|---|---|
| **Protein synthesis and mRNA translation** | | | | | | |
| Elongation factor 1 alpha 1 | 9.1 | 50.08 | P10126 | - | 5 (72) | + 8.8 |
| Elongation factor 2 | 6.4 | 95.12 | P58252 | - | 5 (123) | - 9.1 |
| Eukaryotic initiation factor 4A-II (E.C. 3.6.1.-) | 5.3 | 46.12 | P60843 | - | 1 (37) | + 8.7 |
| Poly(rC)-binding protein | 6.6 | 37.47 | P60335 | - | 3 (139) | + 9.2 |
| T-complex protein 1 | 5.7 | 59.58 | P11984 | - | 6 (320) | - 9.0 |
| **mRNA transcription** | | | | | | |
| Heterogeneous nuclear ribonucleoprotein A/B | 7.6 | 30.81 | Q99020 | - | 1 (41) | - 2.7 |
| Heterogeneous nuclear ribonucleoprotein A2/B1 | 8.6 | 35.97 | O88569 | - | 3 (151) | + 2.7 |
| Heterogeneous nuclear ribonucleoprotein A3 | 9.1 | 39.63 | Q8BG05 | - | 1 (45) | + 2.7 |
| Heterogeneous nuclear ribonucleoprotein H | 5.8 | 49.03 | O35737 | - | 2 (78) | - 6.5 |
| Heterogeneous nuclear ribonucleoprotein L | 9.8 | 60.18 | Q8R081 | - | 1 (36) | - 3.0 |
| **Carbohydrate metabolism** | | | | | | |
| Aldose reductase (E.C. 1.1.1.21) | 6.7 | 35.57 | P45376 | - | 1 (53) | - 8.0 |
| Alpha enolase (E.C. 4.2.1.11) | 6.5 | 46.98 | P17182 | - | 8 (454) | - 6.1 |
| Dihydrolipoyl dehydrogenase, mitochondrial precursor (E.C. 1.8.1.4) | 8.6 | 54.21 | O08749 | - | 1 (40) | - 2.8 |
| Fructose-bisphosphate aldolase (E.C. 4.1.2.13) | 8.4 | 39.19 | Q54AI4 | - | 3 (120) | + 2.7 |
| Gamma enolase (E.C. 4.2.1.11) | 4.9 | 47.13 | P17183 | - | 1 (40) | - 8.5 |
| Glyceraldehyde-3-phosphate dehydrogenase (E.C. 1.2.1.12) | 8.5 | 35.66 | P16858 | - | 4 (176) | - 2.7 |
| Isocitrate dehydrogenase (E.C. 1.1.1.42) | 6.3 | 39.63 | Q9D6R2 | - | 2 (70) | - 2.0 |
| Pyruvate kinase (E.C. 2.7.1.40) | 6.1 | 56.41 | P52480 | - | 1 (36) | - 9.8 |
| Transketolase (E.C 2.2.1.1) | 7.2 | 67.58 | P40142 | - | 1 (37) | - 7.0 |
| Triosephosphate isomerase (E.C. 5.3.1.1) | 7.1 | 26.56 | P17751 | - | 2 (101) | - 2.3 |
| **Proteolysis** | | | | | | |
| Proteasome subunit beta type 7 precursor (E.C. 3.4.25.1) | 7.7 | 29.91 | P70195 | - | 3 (154) | + 2.3 |
| **DNA replication and cell proliferation** | | | | | | |
| DNA licensing factor MCM7 | 5.9 | 81.16 | Q61881 | - | 1 (40) | + 2.3 |
| Dynein light chain 2, cytoplasmic | 6.8 | 10.48 | Q9D0M5 | - | 1 (56) | - 2.5 |
| Histone H2A | 10.8 | 14.04 | P22752 | - | 2 (73) | - 7.5 |
| Histone H2B F | 10.3 | 13.79 | P10853 | - | 3 (108) | - 8.3 |
| Histone H4 | 11.3 | 11.22 | P62806 | - | 1 (42) | - 8.9 |
| Nucleolin (protein C23) | 6.1 | 76.59 | P09405 | 35% (56) | 1 (30) | - 9.0 |
| Peroxiredoxin 1 (E.C. 1.11.1.15) | 8.2 | 22.16 | P35700 | - | 6 (234) | + 2.0 |
| Peroxiredoxin 4 (E.C. 1.11.1.15) | 6.7 | 31.03 | O08807 | - | 1 (45) | + 2.0 |
| Proliferation associated nuclear element protein 1 | 7.7 | 20.04 | Q9CQA0 | 16% (46) | - | - 7.9 |
| **ATP synthesis and respiratory chain** | | | | | | |
| ATP synthase alpha chain, mitochondrial precursor (E.C. 3.6.3.14) | 9.2 | 59.72 | Q03265 | - | 5 (292) | - 9.3 |
| ATP synthase beta chain, mitochondrial precursor (E.C. 3.6.3.14) | 5.1 | 56.26 | P56480 | - | 9 (477) | - 9.8 |

| Protein name | Theorical pI | Theorical Mw (kDa) | Accession number (SwissProt) | % of sequence coverage (MASCOT score) by MALDI-MS | Peptide number (MASCOT score) by LC-ESI-MS-MS | Fold change (adapted/non-adapted) |
|---|---|---|---|---|---|---|
| | | | Protein folding | | | |
| 60 kDa heat shock protein, mitochondrial precursor | 5.9 | 60.91 | P63038 | - | 4 (150) | - 7.7 |
| 78 kDa glucose-regulated protein precursor | 5.1 | 72.37 | P20029 | - | 13 (721) | + 8.7 |
| Protein-disulphide isomerase A3 precursor (E.C. 5.3.4.1) | 5.9 | 56.58 | P27773 | - | 7 (292) | - 4.2 |
| | | | Cell cycle and cell cycle regulation | | | |
| Heat shock cognate 71 kDa protein | 5.4 | 70.82 | P63017 | - | 7 (318) | - 3.2 |
| Heat shock protein 75 kDa, mitochondrial precursor | 6.2 | 80.16 | Q9CQN1 | - | 1 (98) | - 9.0 |
| Heat shock protein 90 alpha | 4.9 | 84.60 | P07901 | - | 1 (57) | - 8.0 |
| Heat shock-related 70 kDa protein 2 | 5.5 | 69.69 | P17156 | - | 2 (109) | - 7.6 |
| Prohibitin | 5.5 | 29.80 | P67778 | - | 6 (253) | + 6.1 |
| Ras-related protein Ral B | 6.2 | 23.34 | Q4KMA9 | 17% (50) | - | + 3.5 |
| Tumour protein p53-inducible nuclear protein 2 | 6.3 | 23.98 | Q8CFU8 | 50% (67) | - | - 8.2 |
| | | | Lipid metabolism | | | |
| Fatty acid-binding protein | 6.1 | 15.12 | P04117 | - | 5 (330) | + 2.9 |
| Low-density lipoprotein receptor-related protein 2 precursor | 5.0 | 518.93 | P98158 | 44% (36) | - | + 5.0 |
| | | | Membrane transport and cytoskeleton structure | | | |
| Actin, cytoplasmic 1 (beta actin) | 5.3 | 41.71 | P60710 | - | 2 (73) | + 2.0 |
| Alpha actin | 5.2 | 41.98 | P68134 | - | 1 (39) | + 2.5 |
| Cofilin-1 | 8.2 | 18.42 | P18760 | - | 2 (121) | - 8.7 |
| Potassium channel subfamily K member 15 | 9.9 | 35.65 | Q8R5I0 | 15% (50) | - | - 8.0 |
| Rab GDP dissociation inhibitor beta-2 | 5.9 | 50.50 | Q61598 | - | 1 (58) | - 9.8 |
| Tubulin beta 3 chain | 4.8 | 50.41 | Q9ERD7 | - | 1 (39) | + 3.2 |
| Tubulin beta 1 chain | 4.7 | 49.63 | P69893 | - | 4 (177) | + 4.0 |
| Tubulin beta-2C chain | 4.8 | 49.83 | P68372 | - | 2 (77) | + 3.5 |
| Voltage-dependent anion-selective channel protein 1 (VDAC -1) (rVDAC1) | 8.6 | 30.62 | Q9Z2L0 | - | 2 (95) | - 9.0 |
| | | | Other functions | | | |
| D-3-phosphoglycerate dehydrogenase (E.C. 1.1.1.95) | 6.1 | 56.41 | Q61753 | - | 5 (290) | - 7.7 |
| Endoplasmic reticulum protein ERp29 precursor | 5.9 | 28.81 | P57759 | - | 1 (37) | - 2.0 |
| IgE-binding protein | 9.4 | 62.71 | P16110 | - | 1 (43) | - 7.8 |
| Stomatin-like protein 2 | 8.9 | 38.36 | Q99JB2 | - | 2 (153) | - 7.2 |
| Superoxide dismutase (E.C. 1.15.1.1) | 4.9 | 15.81 | P08228 | 12% (38) | 1 (30) | + 2.6 |
| Ubiquitin-like protein SMT3A | 5.3 | 10.86 | Q9Z172 | - | 1 (71) | - 8.4? |

and alpha and beta actin (+ 2.5 and + 2.0 fold respectively) were up-regulated in the adapted cells (Table 2).

Western blotting analysis was used to verify two proteins with differential expression. Figures 3A and 3B confirm the down regulation of prohibiting and up regulation of the dyeing light chain after adaptation, respectively.

## Changes in protein expression in Mab-producing NS0 cell lines

Two Mab-expressing NS0 cell lines (NS0/hR3 and NS0/T1hT) were included in this study to analyze whether serum adaptation-dependent changes in protein expression with the same cell lines were expressing different heterologous proteins [18, 19].

In both cases, the majority of detected spots were downregulated in adapted cells, consistent with previously obtained results in the host NS0 cell line (Table 3). However, proteins related to the same processes such as heat shock cognate 71 kDa protein and prohibitin, were found to be differentially expressed between the 3 cell lines.

In total, 36 spots were identified as being differentially regulated with a ≥2 fold change in all 9 sets of 2D gels from the non-adapted group compared to the adapted group, 27 of which were characterized by MALDI-MS and/or LC-ESI-MS-MS (Table 3).

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## *D*iscussion

This study uses 2DE to characterize changes in the proteome upon the adaptation of the NS0 myeloma cell line following its adaptation to the protein-free medium. The identified proteins were grouped according to their molecular function (Figure 4). Three major cellular pathways seem to be involved in the adaptation to the protein-free medium: i) carbohydrate metabolism and energy production, especially glycolysis and the Krebs cycle, ii) protein synthesis and folding; iii) membrane transport, and iv) cell proliferation accounting for 21%, 14%, 15% and 15% of the total identified protein respectively. The possible relevance of those changes for cellular physiology is discussed below.

Six proteins related to glycolysis were identified in this study; most of them were down-regulated in adapted cell lines. This down-regulation could be caused by two different effects: either cell lines cultured in a serum supplemented medium need more metabolic precursors dedicated to cellular growth and viability, or more energy is required for the same objectives. As shown in Table 1, the main difference between the adapted and non adapted cell lines is the maximum viable cell concentration ($Xv_{max}$), suggesting the possible influence of different FBS nutrients in cell growth. Significant differences in specific growth rate (μ) were not found between adapted and non adapted cell lines.

Glycolysis is one of the most important metabolic pathways providing a source of precursors and energy for the cell. A previous analysis by DNA microarray studies has revealed a large number of genes involved in glycolysis, the pentose phosphate pathway and the Krebs cycle to be down regulated in the host NS0 cell line cultured in the absence of cholesterol [35].

Alpha enolase, a metalloenzyme that catalyzes the dehydratation of 2-phospho-D-glycerate (PGA) to phosphoenolpyruvate (PEP) was identified as being down-regulated following adaptation. This is consistent with the overall decrease in thr expression profile of metabolic genes reported by Seth *et al.* [35]. Isocitrate dehydrogenase is also down-regulated after adaptation to the protein-free medium. This protein is of particular importance as it is one of the key regulatory points in the Krebs cycle and it also affects other cellular pathways, providing metabolic precursors. Isocitrate accumulation could provoke a decrease in the rate of the Krebs cycle by its interaction with isocitrate dehydrogenase, resulting in a increased rate of lipid synthesis [36].

Chaperones, especially heat shock proteins (HSP), represent a group of highly conserved protein species which are localized in different cellular compartments and assist newly synthesized proteins in folding or translocating through membranes, stabilizing certain protein conformations and helping to eliminate denatured proteins [37]. Chaperones also play a role within the regulatory network of the cell cycle and within signal cascades. Synthesis of chaperones/HSPs is induced in the presence of serum, mitogens or growth factors [38-40].

Chaperones and associated proteins such as the 60 kDa heat shock protein, the heat shock cognate 71 kDa protein and the heat shock-related 70 kDa

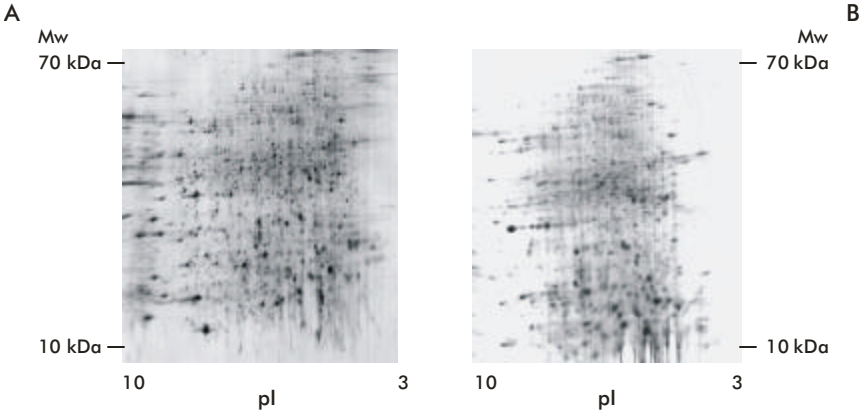


Figure 3. Representative silver-stained 2D gels corresponding to Mab-producing NS0 myeloma cell lines adapted to a protein-free medium. The NS0/hR3 cell line adapted (A) and non-adapted (B).

Table 3. Comparison of changes in expression of identified proteins following adaptation to a protein-free medium of host and recombinant NS0 cell lines. (*): protein not identified by MS, (+): up-regulated proteins, (-): down-regulated proteins

| Protein name | Change in expression (non-adapted/adapted cell line to a protein-free medium) | | |
|---|---|---|---|
| | Host NS0 cell line | NS0/hR3 cell line | NS0/hT1hT cell line |
| 60 kDa heat shock protein, mitochondrial precursor | + | - | * |
| 78 kDa glucose-regulated protein precursor | - | + | * |
| Alpha enolase | + | + | + |
| ATP synthase beta chain, mitochondrial precursor | + | + | * |
| D-3-phosphoglycerate dehydrogenase | + | + | * |
| Dynein light chain 2, cytoplasmic | + | + | * |
| Elongation factor 1 alpha 1 | - | - | * |
| Elongation factor 2 | + | + | * |
| Fatty acid-binding protein | - | - | * |
| Fructose-bisphosphate aldolase | - | - | * |
| Glyceraldehyde-3-phosphate dehydrogenase | + | + | + |
| Heat shock cognate 71 kDa protein | + | - | + |
| Heat shock-related 70 kDa protein 2 | + | + | * |
| Heterogeneous nuclear ribonucleoprotein H | + | + | * |
| Heterogeneous nuclear ribonucleoprotein L | + | + | + |
| Histidine triad nucleotide-binding protein 1 | * | - | - |
| Peroxiredoxin 1 | - | - | * |
| Peroxiredoxin 4 | - | - | * |
| Poly(rC)-binding protein | - | - | * |
| Potassium channel subfamily K member 15 | + | * | + |
| Prohibitin | - | + | - |
| Pyruvate kinase | + | + | * |
| Rab GDP dissociation inhibitor beta-2 | + | - | * |
| Triosephosphate isomerase | + | - | * |
| Tubulin beta 1 chain | - | - | * |
| Tumor protein p53-inducible nuclear protein 2 | + | * | + |
| Voltage-dependent anion-selective channel protein 1 (VDAC-1) (rVDAC1) | + | + | + |

protein 2 were found to be down-regulated after the adaptation to a protein-free medium in the host NS0 cell line and the NS0/hT1hT cell line as expected. Interestingly, the heat shock cognate 71 kDa protein was found to be up-regulated after adaptation in the NS0/hR3 cell line. Other heat shock proteins that varied their expression level ratio before and after adaptation in the NS0/hR3 cell line, with respect to the host NS0 cell line and the NS0/hT1hT cell line were prohibitin and the 78 kDa glucose-regulated protein precursor. These results are related to the difference shown in $Xv_{max}$ between the host NS0 cell line and the NS0/hR3 cell line (Table 1). The host NS0 and NS0/hT1hT cell lines decreased their $Xv_{max}$ value after adaptation, indicating a possible decrease in protein synthesis and cell proliferation rate. Contrarily, the $Xv_{max}$ of the NS0/hR3 cell line increased after adaptation to the serum free medium. In this case, the change in the Xvmax value could be related to the introduction of a new protein expression system, indicating that the energy and protein synthesis ability in this condition could be dedicated to anti-EGF Mab production. These changes suggest a possible relationship between these proteins and Mab expression during adaptation to the protein-free medium, particularly in a case of the NS0/hR3 cell line.

The proteins involved in the transport through the cytoplasmic membrane were also identified as being differentially regulated following adaptation. Proteins such as the potassium channel subfamily K member 15 and the voltage-dependent anion-selective channel protein 1 decreased their expression level after adaptation, suggesting that the adapted cell lines needed less activity from these proteins. Serum is formed by several proteins and other supplements needed for cell growth as previously mentioned. The cells cultured under these conditions need an over-expression of proteins related with membrane transport in order to use the serum molecules during growth and metabolism.

The LDL-receptor related protein 2 precursor was up-regulated (5.0 fold) after adaptation. Previous studies have reported the importance of LDL for the serum-free growth of NS-1 myeloma cells. LDL (or HDL) promotes NS-1 myeloma growth by supplying cholesterol, since cholesterol, when added under the appropriate conditions, mimics LDL in promoting NS-1 myeloma cell growth [41]. This up-regulation in adapted cells suggests an increase in the activity of this protein during the adaptation process.

In contrast with membrane transport proteins, many proteins related to the cytoskeleton structure increased their expression level after adaptation. These include several tubulin isoforms, beta 1, 2C and 3 chain (4.0, 3.2 and 3.5 fold, respectively), as well as alpha and beta actin (2.5 and 2.0 fold, respectively), suggesting a change in cytoskeleton structure. This is consistent with previously reported studies [42] and may account for the decrease in $Xv_{max}$ (Table 1) due to a loss of the anchorage dependency of these cell lines after the adaptation to the protein-free medium.

Rab GDP dissociation inhibitor beta 2 was found to be down-regulated after its adaptation in the host NS0 cell line, while it was up-regulated after its adaptation in the NS0/hR3 cell line. GDP dissociation inhibitors (GDI) are proteins that regulate the GDP-GTP exchange reaction for members of the Rab family, which serve a regulatory role in membrane traffic. Rab proteins are in part cytosolic and in part associated with the membranes of specific exocytic and endocytic organelles [42-44]. GDI may play a role in cholesterol uptake or trafficking inside the cell.

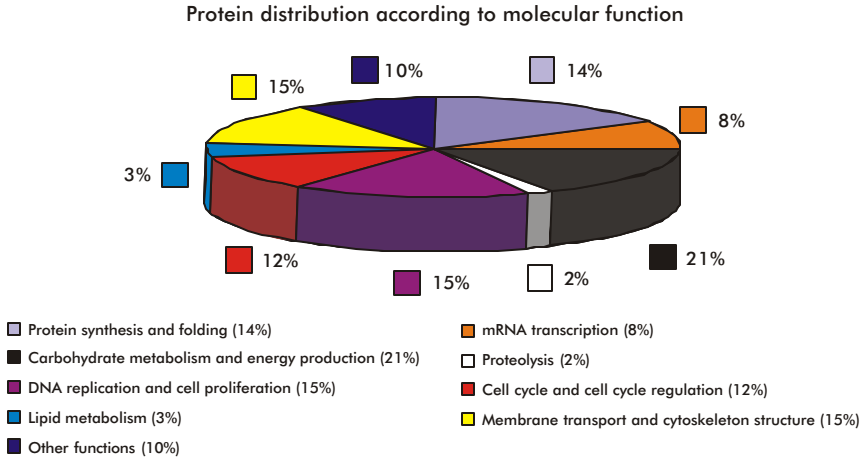


Figure 4. Distribution of differentially regulated proteins (following adaptation) in the host NS0 cell line according their molecular function.

### Other cellular processes related to adaptation to a protein-free medium

Several authors have reported changes in the expression levels of proteins involved in the cell cycle and DNA replication following the adaptation of CHO cells to a protein-free medium [10, 45, 46]. Proteins in this class, including peroxiredoxin 4, dynein light chain 2, prohibitin, heat shock cognate 71 kDa protein and tumour protein p53-inducible nuclear protein 2 were also identified in this study.

DNA microarray studies reported by Seth *et al.* [35], demonstrated down regulation in a number of genes in host NS0 cells grown in the absence of cholesterol in the medium, but they did not find any changes in expression after 2DE and MS analysis. In accordance with the paper, none of the differentially expressed proteins identified in this study were involved directly in cholesterol metabolism. However, significant intracellular levels of cholesterol in adapted cell lines were found, indicating that this cell line lost its auxotrophy to cholesterol after its adaptation to the protein-free medium.

## Conclusions

The analysis presented provides an overview of the major molecular pathways involved in the adaptation of NS0 myeloma cell lines to growth in a protein-free medium.

In this study we observed a total of 260 protein spots which exhibited expression changes after their adaptation to a protein free medium; 160 of these were identified by MS. They correspond to proteins related to carbohydrate metabolism and energy production, protein synthesis and folding, membrane transport and cell proliferation.

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Significant intracellular levels of cholesterol were found in the adapted cell line, demonstrating that the pathways involved in cholesterol metabolism were activated after their adaptation to the protein-free medium. Consistent with previous results that detected changes in mRNA expression related to cholesterol metabolism but not protein expression, no proteins directly related with cholesterol synthesis were found in this study. These proteins may be expressed at levels that are too low to be detected under these conditions, they may exhibit less than a 2-fold change, or they may be present in the membrane fraction and are therefore not recognised using the conventional 2DE analysis performed here.

By comparing the host NS0 cell line and two derived lines expressing Mabs, it was possible to discriminate changes in protein expression related to Mabs expression from those related to changes due to adaptation to a protein-free medium. Some of these proteins behaved differently in the two transformed lines, indicating that the exogenous antibody expression affects adaptation of the cell line to a protein-free medium.

*Received in September, 2007. Accepted for publication in December, 2007.*