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ARTICLE

Enhanced production and isotope enrichment of recombinant glycoproteins produced in cultured mammalian cells

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Abstract NMR studies of post-translationally modified proteins are complicated by the lack of an efficient method to produce isotope enriched recombinant proteins in cultured mammalian cells. We show that reducing the glucose concentration and substituting glutamate for glutamine in serum-free medium increased cell viability while simultaneously increasing recombinant protein yield and the enrichment of non-essential amino acids compared to culture in unmodified, serum-free medium. Adding dichloroacetate, a pyruvate dehydrogenase kinase inhibitor, further improves cell viability, recombinant protein yield, and isotope enrichment. We demonstrate the method by producing partially enriched recombinant Thy1 glycoprotein from Lec1 Chinese hamster ovary (CHO) cells using U-^{13}C-glucose and ^{15}N-glutamate as labeled precursors. This study suggests that uniformly ^{15}N,^{13}C-labeled recombinant proteins may be produced in cultured mammalian cells starting from a mixture of labeled essential amino acids, glucose, and glutamate.

Keywords Recombinant glycoprotein · Isotope enrichment · Mammalian cells · NMR · Thy1

Introduction

NMR studies of glycosylation or other post-translational modification effects on protein structure, function, and dynamics may require recombinant protein expression in eukaryotic cells. Producing high levels of highly enriched recombinant proteins in mammalian cells is particularly challenging because mammalian cells do not synthesize all 20 proteinaceous amino acids and because they efficiently metabolize glucose into lactate. To circumvent these problems, Hansen et al. developed a labeling strategy where isotope enriched amino acids were introduced into the culture medium (Hansen et al. 1992); a similar strategy is followed in insect cells (Brüggert et al. 2003; Strauss et al. 2003, 2005; Walton et al. 2006). However, only a few reports have pursued this approach for producing isotope enriched recombinant proteins (Archer et al. 1993; Lustbader et al. 1996; Wyss et al. 1993, 1995). Despite the introduction of commercially available algal biomass and labeled growth medium supplements, poor reproducibility and the high cost of the isotope enriched medium have limited the utility of eukaryotic cells for investigating the structural biology of glycoproteins, in particular, and of post-translationally modified proteins, in general.

CHO cells are commonly used for producing recombinant (glyco)proteins. An extensive literature exists on optimizing cell viability and recombinant protein production in these and other cultured mammalian cells, but the utility of these methods in enhancing isotope incorporation has not been examined. Fundamentally, the challenge is to produce the highest amount of isotope enriched recombinant protein

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per liter of growth medium. Here we identify simple modifications to commercially-available serum-free growth medium (SFM) that significantly increases cell viability and recombinant protein production while reducing the production of toxic metabolites. Furthermore, these modifications increase the isotope enrichment of non-essential amino acids that are incorporated into recombinant glycoproteins. These results suggest a straightforward approach to producing partially or fully isotope enriched recombinant proteins in mammalian cells for NMR investigations.

Materials and methods

Serum-free medium (HyQ SFM4CHO), glucose-free DMEM, and cosmic calf serum were from Hyclone (Logan, UT). DMEM with glutamine, without ribonucleosides and deoxyribonucleosides, sodium butyrate and dichloroacetic acid were from Sigma (St. Louis, MO). U-^{13}C-glucose, U-^{13}C-glutamate and ^{15}N-glutamate were from Cambridge Isotopes (Andover, MA). Q-Sepharose resin, Concanavalin A resin, and Thrombin were from GE Biosciences (Piscataway, NJ). Penicillin–Streptomycin and Gentamicin were from Invitrogen/Gibco (Carlsbad, CA). All other materials and reagents were cell culture grade from Fisher Scientific (Hampton, NH) unless otherwise indicated.

Growth medium optimization and cell assays

Lec1 CHO cells expressing a secreted Thy1-GFP fusion construct (Mehndiratta et al. 2004) were grown to ~80% confluency in a T-225 flask in the presence of high-glucose DMEM (4.5 g/L glucose) containing 10% Cosmic calf serum, 40 mg/L proline, and Penicillin–Streptomycin-Gentamicin antibiotics. These cells were then plated at a density of 1.6×10^4 cells per cm^2 in 6-well plates and kept at 37°C under 5% humidity. After 48 h the cells were washed twice with phosphate-buffered saline (PBS) and switched to 2 mL of the indicated medium.

Cell count and viability assay

Cell counts and viability were determined using a Cedex HiRes cell analyzer (Innovatis AG Inc, Malvern, PA) according to the manufacturer's protocol. Briefly, spent medium was aspirated and the cells washed twice with PBS. Adherent cells were suspended by trypsinization (1 mL of TrypLE Express (Invitrogen/Gibco, Carlsbad, CA) per 10 cm^2) and combined with 1 mL of serum-containing medium to stop trypsin activity, from which 300 μL was sampled in the Cedex HiRes cell analyzer.

Glucose assay

Medium glucose concentration was determined using the GlucCell glucose meter with GlucCell glucose test strips (Bellco Glass Inc., Vineland, NJ) according to the manufacturer's protocol.

Thy1 expression assay

Secreted Thy1-GFP was measured in medium clarified by centrifuging at 800*g* for 10 min at 4°C. Fluorescence was measured using a NanoDrop spectrofluorometer (NanoDrop Technologies Inc., Wilmington, DE) with excitation at 470 nm and emission at 510 nm using 10 nm slit widths and a gain of 1.00.

Metabolite extraction and derivatization of Lec1 CHO cells

Cells from 10 cm^2 plates were harvested by washing twice with PBS, suspended with trypsin, and pelleted at 800*g* for 10 min at 4°C. The cell pellet was washed again with PBS and then extracted by adding 500 μL of 100% methanol plus 5 μL internal standard (2 mg/mL norleucine in water) with heating at 70°C for 15 min (Roessner et al. 2000). The extract was cooled to room temperature and one volume of H_2O was added with vigorous mixing. Insoluble material was pelleted and the clarified supernatant dried under vacuum overnight in a 1 mL reaction vial. Dried extracts were dissolved in 20 μL dimethylformamide and 40 μL N-methyl-N-(*tert*-butyldimethylsilyl)trifluoro-acetamide containing 1% *tert*-butyldimethylchlorosilane (Thermo Scientific, Rockford, IL). The reaction vial was sealed under nitrogen and heated at 75°C for 30 min (Mawhinney et al. 1986).

GC–MS analysis of metabolite extracts

Derivatized metabolite samples (1 μL) were injected in splitless mode into an HP Agilent 6890 series gas chromatograph coupled with an HP Agilent 5973 mass selective detector and separated on a 30 m DB-5 column (J&W Scientific, Folsom, CA) (Wittmann et al. 2002). Mass spectra were collected at a rate of 2 Hz over a scanning range of *m/z* 50–650. Metabolites were identified by comparison with standards; unknowns were identified from their retention time and mass spectrum by searching the NIST02 mass spectral library using tools available in Wsearch32 (www.wsearch.com.au) and confirmed using authentic samples. Absolute peak areas for each metabolite were normalized to the absolute peak area of the internal standard norleucine and then further normalized to cell count.

Calculation of isotope incorporation

Mass isotope distribution vectors (MID) were calculated according to published procedures (Lee et al. 1991; Jennings and Matthews 2005; Van Winden et al. 2002) using routines written in R or MATLAB and interfaced with python scripts that parsed the chromatograms and mass spectra (scripts available from the authors upon request). Briefly, the abundance of a given isotopomer for a chosen ion in the mass spectrum was calculated using a binomial distribution assuming that isotopes of carbon, nitrogen, and silicon are the only significant contributors to the MID (abundances for these nuclei were from IUPAC). The natural abundance isotope distribution matrix was generated to incorporate up to n ^{13}C atoms and m ^{15}N atoms, where n (m) is the number of carbons (nitrogens) in a given amino acid, correcting the probabilities for successive values of n (m) as described by Jennings and Matthews (2005). The abundance matrix, A, was used to estimate the populations of each isotopomeric species, x, given the observed mass spectrum intensities, y, as (Brauman 1966)

$$x = (A^T A)^{-1} A^T \cdot y.$$

MID were calculated for the molecular ion and several fragment ions typically observed for tert-butyl-dimethylsilyl (tBDMS) derivatives of amino acids (Mawhinney et al. 1986).

Large-scale growth of Lec1 cells

Lec1 cells were expanded to confluency in standard 800 cm^2 roller bottles in DMEM prior to switching to the desired expression and labeling medium. Our previous culture protocol (Mehndiratta et al. 2004) was modified by reducing the culture volume to 50 mL in each roller bottle, exchanging spent for fresh medium after 48 h and then every 96 h subsequently. Reducing the medium volume in each roller bottle increased recombinant protein yield per liter ~20-fold compared to our previous work (Mehndiratta et al. 2004) due to increased oxygenation and reduced mechanical disruption of the monolayer.

NMR spectroscopy

Thy1 was purified from the growth medium and GFP proteolytically released as described previously (Mehndiratta et al. 2004; Walton et al. 2006). Purified Thy1 was dried and reconstituted in 25 mM potassium phosphate buffer, pH 6.8 containing 10% D_2O. 2D ^1H-^{13}C HSQC spectra were collected on a 500 MHz Bruker Avance spectrometer as 1,024 × 72 complex points over 8,333.33 and 20,833.33 Hz sweep widths in the ^1H and ^{13}C dimensions, respectively, using a sensitivity enhanced HSQC pulse sequence (Kay et al. 1992; Palmer et al. 1991; Schleucher et al. 1994). A 2D ^1H,^{15}N-HSQC spectrum (Mori et al. 1995) was collected on a 900 MHz Varian Inova console as 1,024 × 128 complex points over 14,534.9 and 3,600 Hz sweep widths in the ^1H and ^{15}N dimensions, respectively.

Results

Glucose atoms are incorporated into non-essential amino acids

Our initial objective was to isotopically enrich the carbohydrates on recombinant Thy1 produced in Lec1 cells for NMR studies. Thy1-producing Lec1 cells were expanded to confluency in 10 roller-bottles at which point glucose in the SFM was switched to U-^{13}C-glucose at the same concentration. The cells were grown in this medium for 96 h with one medium change at 48 h, yielding a 150 μM Thy1 sample. Recombinant protein was not collected beyond 96 h due to significant cell death and increased glycosylation defects. This protocol yielded intense labeling of carbohydrate resonances in 2D ^1H,^{13}C HSQC spectra of purified Thy1 (Supplemental Figure 1), many of which could be assigned to resonance type by inspection based on comparison with assignments for carbohydrate resonances in other glycoproteins (Wyss et al. 1995). We also observed label incorporation into resonances that are associated with amino acids. Labeling of these amino acid resonances was increased by growing the cells to confluency in U-^{13}C-glucose enriched DMEM before switching into U-^{13}C-glucose enriched SFM (total exposure to labeled precursors was ~144 h), but this required significantly larger culture volumes and correspondingly larger amounts of labeled glucose and was abandoned as being cost prohibitive.

Altering the metabolic profile of CHO cells

We then investigated how to produce the most recombinant protein per liter of growth medium while simultaneously increasing the isotope enrichment of the non-essential amino acids. The primary limitation to using SFM (and serum-containing medium as well) for this objective is the high rate of lactate and ammonium ion production (Irani et al. 1999). As with other highly proliferative cells (Vander Heiden et al. 2009), CHO cells efficiently funnel glucose through glycolysis but produce lactate rather than continue metabolism via the TCA cycle, possibly due to reduced activity in pyruvate dehydrogenase. Commercial growth media based on Eagles original formulations (Eagle 1955, 1959) contain high levels of glutamine (5 g/L or

35 mM) to overcome this metabolic bottle-neck, but slow hydrolysis of the amido nitrogen increases NH_4^+ concentration in the medium. The high lactate and NH_4^+ levels severely limit cell viability and frequent medium changes (every 48 h) are required to maintain lactate and ammonium at sub-toxic levels and to fuel the rapid proliferation of these cells.

Numerous studies have shown that reducing lactate and ammonium levels increase cell viability and may increase recombinant protein production. For example, Altmamirano and co-workers (Altamirano et al. 2000, 2001a) showed that simultaneous replacement of glucose by galactose and glutamine by glutamate in CHO cells effectively eliminated lactate and ammonium production while increasing and prolonging cell viability and recombinant protein yield (Altamirano et al. 2000, 2001a). In our hands, this double metabolite replacement strategy resulted in severe morphology changes that are typical of glucose- and glutamine-deprived cells (Zanghi et al. 1999; Fumarola et al. 2001; Paquette et al. 2005; Tzatsos and Tsichlis 2007) and extensive cell death. Prolonged culture in this medium might have allowed cells to adapt to this medium as found by Altimirano, but the lack of commercially available U-^{13}C-galactose reduces the utility of this approach as a cost-effective strategy for producing isotope enriched recombinant glycoproteins.

We then assayed for consumption of glucose in the SFM under normal cell culture conditions. The CHO cells consumed <2 g/L glucose over 48 h, leaving >5 g/L in the discarded medium. Based on this, we reduced the amount of glucose (to 2 g/L; 11 mM) and replaced glutamine with glutamate (at 1 g/L; 7 mM) in the SFM. We refer to this as a reduced nutrient (RN) SFM medium. In a separate set of experiments, we added dichloroacetate (DCA) to the reduced nutrient serum-free medium. DCA inhibits pyruvate dehydrogenase kinase, a negative regulator of pyruvate dehydrogenase (Bowker-Kinley et al. 1998), and is used to stimulate recoupling of glycolysis and TCA cycle under a variety of conditions (Lloyd et al. 2003; Michelakis et al. 2008).

Figure 1a compares the cell morphology of Lec1 cells at 48 h after introduction of either SFM or RN growth medium. Cells were expanded to confluency in 6-well plates in DMEM and then changed to the indicated medium. After only 48 h the SFM-treated cells showed signs of severe stress as evidenced by the spindle shape of the attached cells and by the clumping and sloughing off of large numbers of cells. The RN-treated cells were highly confluent and had a normal morphology at the same time point; DCA-treated cells were identical to the RN-treated cells (not shown). At longer time points, the SFM cells appeared severely stressed with few remaining viable cells. The RN and DCA-treated cells remained highly confluent but had an elongated, spindle morphology and appeared to grow in tracts. Cells could be cultured for 600 h (longest time point

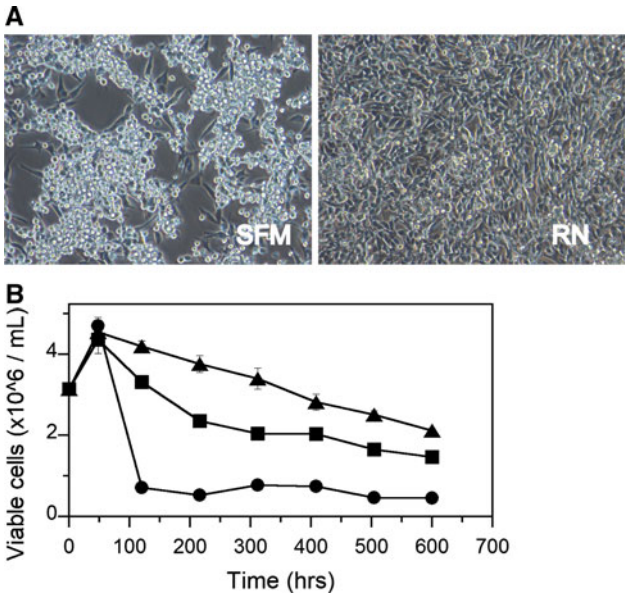

Fig. 1 Reduced nutrient medium formulations show enhanced cell morphology and increased cell viability. **a** Phase contrast micrograph of Lec1 cells grown in SFM and in RN for 48 h. Cells grown in DCA were identical to those grown in RN (not shown). **b** Number of viable cells in SFM (*filled circle*), RN (*filled square*), and DCA (*filled triangle*) as a function of time in culture

investigated) with minimal change in cell morphology and only slight reductions in cell number in the RN and DCA media. Figure 1b plots the cell viability in the three media formulations over the 600 h time course of the experiment. After a slight increase in the number of viable cells in all media at 48 h (cells were not synchronized), the number of viable cells plummeted in the SFM medium whereas the RN and DCA media maintained significantly higher levels of viable cells. The DCA medium consistently exhibited the highest viability throughout the experiment time course.

Figure 2 plots extracellular glucose and intracellular lactate levels for cells grown in each medium. As anticipated, the SFM-treated cells consumed ~2 g/L of the supplied glucose between medium changes with efficient metabolism of the glucose into lactate, which spiked at ~200 h of culture. The RN- and DCA-treated cells consumed glucose at nearly the same rate as the SFM-treated cells but lactate was significantly reduced in the RN-treated medium and was barely detectable in the DCA-treated cells. These data indicate that the RN and DCA media formulations improve glucose utilization in CHO cells. Figure 2c plots the level of secreted Thy1-GFP from Lec1 cells grown in SFM, RN, and DCA media. SFM-treated cells produced recombinant protein initially at a high rate, peaking at ~100 h but then dropped to very low levels for the remainder of the experiment (consistent with the low number of viable cells). Protein production in the

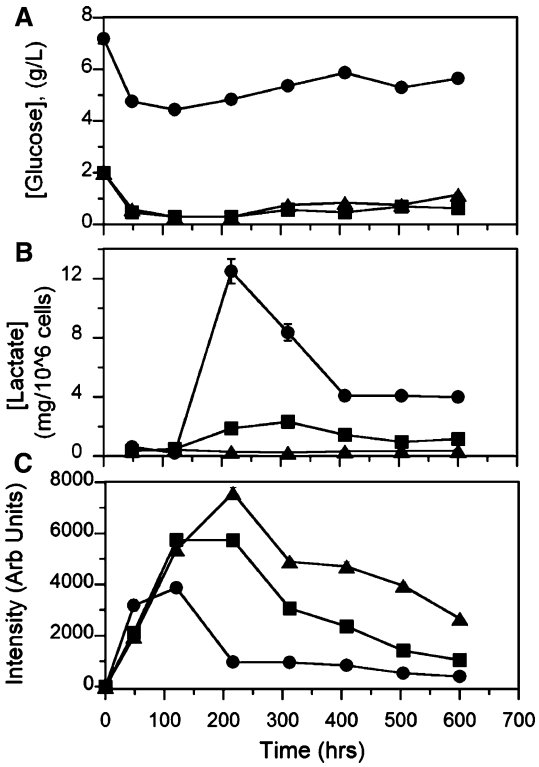

Fig. 2 Reduced nutrient formulations improve glucose utilization without producing lactate and enhance recombinant Thy1 expression. **a** Glucose remaining in the spent medium at the indicated time points. The *zero time point* represents the concentration in fresh medium. **b** Intracellular lactate concentration. **c** Fluorescence intensity due to Thy1-GFP secreted into the growth medium. *Symbols*: *filled circle* SFM, *filled square* RN, *filled triangle* DCA. Data points are the average of three independent measurements and *error bars* are the standard deviation. *Solid lines* are drawn to guide the eye

RN- and DCA-treated cells was initially lower than in the SFM-treated cells, but peaked later (at roughly 175 and 200 h, respectively) and was maintained at a higher total level throughout the remaining experiment time course. After 600 h of culture, the DCA-treated cells produced roughly 300% of the total protein produced in the SFM-treated cells. Interestingly, recombinant protein produced per viable cell remained constant for all three cell lines, with the SFM-treated cells consistently out-performing the RN- and DCA-treated cells on a per cell basis (not shown). Therefore, we investigated whether protein production could be increased in cells cultured in RN or DCA medium following transfer back into SFM. However, cells grown in RN medium for 216 h and then transferred into SFM exhibited a significant reduction in cell viability and a corresponding drop in recombinant protein production to below that seen in cells cultured continuously in RN or DCA medium. The main effect of the RN and DCA formulations, then, is to maintain greater cell viability compared to SFM. Furthermore, the protective effects engendered by the RN and DCA media are dependent upon continued culture in that medium.

Isotope incorporation into non-essential amino acids

We investigated the metabolic fate of labeled glucose by extracting and derivatizing the polar metabolites to form the *t*-butyldimethylsilyl (tBDMS) derivatives, and analyzing label incorporation using gas chromatography-mass spectrometry (GC–MS). Metabolites were tentatively identified based on retention time and mass spectral fragmentation pattern (Mawhinney et al. 1986), and confirmed by comparison with standards. The extraction and derivatization procedure followed in these experiments precluded observation of phosphorylated metabolites but we were able to routinely identify 15 amino acids, several TCA cycle compounds, lactate, urea, palmitate, stearate, and cholesterol (Table 1, Supplementary Material).

Isotope enrichment in the metabolites was calculated following standard methods (Jennings and Matthews 2005; Lee et al. 1991; Van Winden et al. 2002). We first calculated the natural abundance isotope enrichment of the metabolite and tBDMS groups by assuming that only isotopes of carbon, nitrogen, and silicon accounted for the observed isotope distribution and then corrected the intensities for multiple isotope incorporations. The predicted intensities for a given mass spectral peak (the [M-57] + fragment for amino acids (Mawhinney et al. 1986)) were then used to fit the observed mass spectral intensities to obtain the mole fraction of each individual isotopomer.

We observed label incorporation into several non-essential amino acids, including Ala, Ser, Gly (all derived from glycolytic intermediates), Glu and Asp (both derived from TCA cycle intermediates). Figure 3 plots the absolute total molar percent enrichment (AT-MPE) for these five amino acids at all time points obtained from cells exposed to U-^{13}C-glucose in each medium. The AT-MPE is the mole fraction of all isotope enriched species and is a convenient measure of the extent of total label incorporation. For some amino acids, the AT-MPE was dominated by a single isotopomer, such as the $m + 3$ isotopomer in alanine. For others, such as aspartate, there was significant population of the $m + 2$, $m + 3$, and $m + 4$ isotopomers, representing information on the different metabolic sources of the amino acid. We included the $m + 1$ isotopomer population in the AT-MPE calculation although this isotopomer contains a significant contribution from the atoms in the tBDMS groups.

The AT-MPE for all five non-essential amino acids was increased by as much as 50% in the RN and DCA media compared to the SFM medium. The MPE for TCA cycle compounds was also increased in these media (not shown).

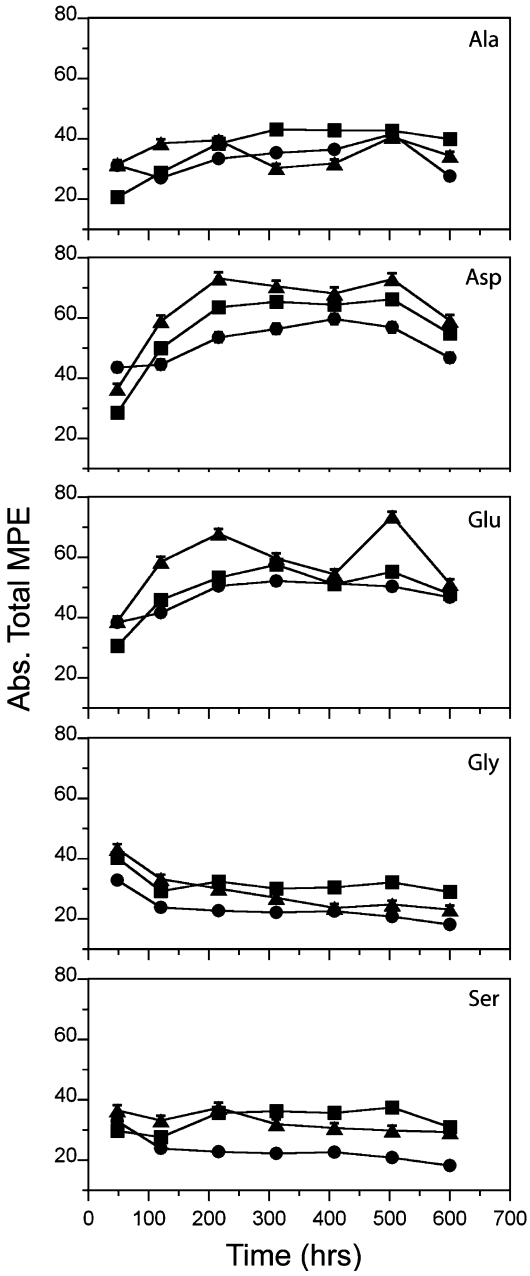

Fig. 3 Absolute total molar percent enrichment (AT-MPE) of 5 non-essential amino acids as a function of growth medium and labeling period. *Key*: *filled circle* SFM, *filled square* RN, *filled triangle* DCA. Each point is the average of three independent measurements; *error bars* represent the standard deviation of the three measurements. *Solid lines* are drawn to guide the eye

Increased enrichment of TCA cycle intermediates and amino acids derived from this cycle may reflect an increased flow of carbons from glycolysis into the TCA cycle in the RN- and, especially, the DCA-treated cells (Ward et al. 1995). Addition of U-^{13}C-glutamate in RN- and DCA-treated cells increased the AT-MPE of Glu and Ala but the AT-MPE of Asp, Ser or Gly was similar to U-^{13}C-glucose labeling alone (not shown).

Nitrogen metabolism in DCA-treated cells

Nitrogen metabolism and labeling in the DCA medium was investigated using ^{15}N-Glu. Nitrogen incorporation (measured as the AT-MPE) was highest for Lys, Asp, and Glu, followed by Ala (Supplemental Figure 2). Detectable, but significantly lower levels of ^{15}N were incorporated into Ser and Gly. Asp, Glu, Ala, Gly, and Ser were also significantly labeled by glucose, indicating that these are the most metabolically active amino acid pools in CHO cells.

Isotope enrichment in recombinant Thy1

Figure 4 shows a 2D ^{1}H,^{13}C HSQC spectrum of Thy1 purified from Lec1 cells grown in DCA-treated medium containing U-^{13}C-glucose and ^{15}N-glutamate. Protein was collected from a single roller bottle between 216 and 600 h in culture with medium changes every 96 h. The final protein concentration was 50 μM isolated from a total of 250 mL of culture medium (0.75 mg/L of culture medium compared with roughly 0.25 mg/L in SFM). Thy1 showed the same electrophoretic mobility as protein isolated previously (Mehndiratta et al. 2004) indicating that the modified medium did not grossly alter glycosylation. The NMR spectrum shows a similar labeling pattern compared with the spectrum of Thy1 produced using SFM (Supplemental Figure 1), but the label incorporation into amino acids is more intense relative to the sugar resonances, exhibiting additional resonances that were not observed previously. Adding protein collected during the initial 216 h of culture

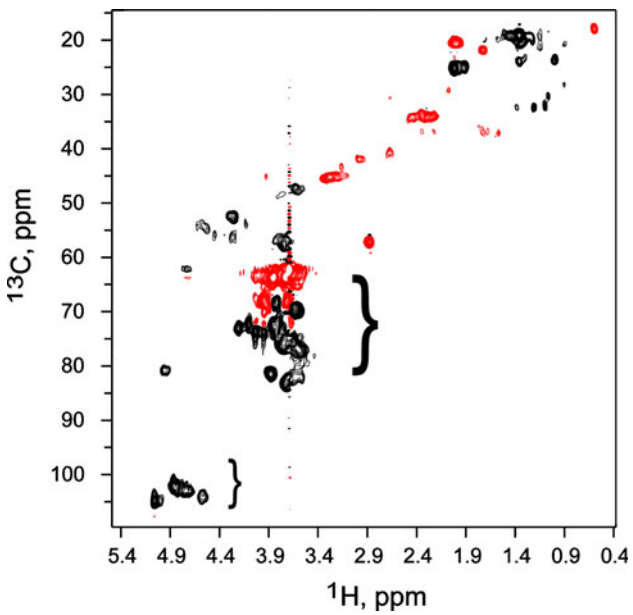

Fig. 4 ^{1}H,^{13}C-HSQC spectrum of Thy1 obtained from Lec1 cells grown in DCA medium containing U-^{13}C-glucose and ^{15}N-glutamate

did not significantly alter the spectrum although sensitivity was improved (not shown).

A $^{1}H,^{15}N$-HSQC spectrum collected on the same sample (Supplementary Figure 2b) demonstrated distribution of the glutamate amino group into other amino acids. The spectrum showed strong intensity in resonances associated with glutamine and asparagine sidechains, consistent with the presence of glutamine synthetase in CHO cells (Altamirano et al. 2001b; Altamirano et al. 2000; Yang and Butler 2000). Otherwise, the overall labeling intensity and distribution into backbone amides was poor, as expected from the relatively weak ^{15}N AT-MPEs detected by GC–MS.

Discussion

Carbon and nitrogen metabolism in CHO cells

Most commercial growth media contain glucose and glutamine as the two major carbon sources. Cultured CHO cells efficiently metabolize medium glucose to pyruvate, producing a net 2 ATP per glucose. Glutamine is metabolized into α-ketoglutarate (yielding two equivalents of NH_4^+) and then into pyruvate via malic enzyme (glutaminolysis), further increasing pyruvate levels. However, metabolism of pyruvate into the TCA cycle is suppressed, resulting in accumulation of lactate and high concentrations of alanine. Lactate and NH_4^+ are toxic to the cultured cells and there is an extensive literature for circumventing this metabolic bottle-neck, including using cells transfected with pyruvate carboxylase (Irani et al. 1999; Elias et al. 2003), and modifying commercial growth medium by reducing glucose and/or glutamine (Öhman et al. 1995; Cruz et al. 2000; Altamirano et al. 2004; Maranga and Goochee 2006). Each of these methods were shown to reduce accumulation of toxic metabolites, prolong cell viability, and increase recombinant protein production, but none were investigated for their effect on isotope labeling of recombinant proteins. Here, we show that reducing glucose and substituting glutamate for glutamine enhances cell viability and increases recombinant protein production in Lec1 CHO cells while also enhancing isotope enrichment of several non-essential amino acids.

We reduced glucose from ~35 mM (7 g/L) to 11 mM (2 g/L) in the culture medium. Significant reduction in cell growth or ATP production in cultured CHO cells is generally not observed until glucose is reduced below 2 mM when glutamine was present (Lu et al. 2005). When extracellular glucose drops below 2 mM, catabolism of glutamine and the branched chain amino acids increases in CHO and other cell types (Martinelle et al. 1998; Öhman et al. 1995; Cruz et al. 2000; Ljunggren and Haggstrom 1994; Li et al. 2005; Zielke et al. 1978). Consumption of the extracellular branched chain amino acids was actually reduced in the RN- and DCA-media compared to SFM (not shown) suggesting that the RN and DCA media do not result in glucose-starved conditions. Therefore, the glutamate/glutamine switch may have greater consequences in cell morphology and metabolism than the reduced glucose.

Eagle showed that glutamine deprivation resulted in rapid loss of cell viability (Eagle 1955, 1959). However, in the 50 years since Eagle's work, an appreciation has developed for the broad role played by glutamine in cellular metabolism as a key regulatory molecule (Curi et al. 2005a, b; Newsholme et al. 2003a, b), including being an allosteric effector for kinases in the PI3 K/Akt/mTOR pathway and amino acid transport systems (Franchi-Gazzola et al. 2006). Glutamine deprivation triggers apoptosis in a variety of cells (Fuchs and Bode 2006; Goswami et al. 1999) through changes in cellular redox status (Criscuolo et al. 2006; Mates et al. 2002) and in mTOR/pS6 K signaling deriving from cell morphology changes (Fumarola et al. 2001, 2005a, b). Consequently, glutamine is an important component of growth media and is a prime concern when formulating isotope enriched growth media (Hansen et al. 1992). Given the ability of CHO cells to synthesize glutamine via glutamine synthetase, we reasoned that the primary metabolic and regulatory roles of glutamine could be substituted by glutamate. We did not observe the drastic morphology and apoptotic effects associated with glutamine removal and found direct evidence that glutamine was being synthesized in the CHO cells. Indeed, protection against the massive cell death observed in RN and DCA-fed cells may be due to the synthesized glutamine.

We investigated whether these protective effects were due to the reduced glucose or to the glutamate substitution by growing cells in low glucose SFM, e.g., glucose at 2 g/L with normal glutamine, or SFM + Glu, e.g., normal glucose concentration but glutamate at 1 g/L and no glutamine. Cells grown in media having one of the two substitutions exhibited cell counts and Thy1 production that was similar to commercial SFM at each time-point but the percent of viable cells was higher in the single-substitution media than in SFM. Interestingly, glucose consumption in SFM + Glu was lower than in SFM, RN- or DCA-treated cells. Glucose consumption in the low-glucose SFM was roughly equivalent to that in the RN or DCA media. Lactate production in the single substitution media was roughly equivalent to that seen in RN medium and much lower than in commercial SFM. From this we conclude that the cell protective effects of the RN and DCA media formulations require both substitutions although the metabolic effects may be additive.

Isotope enrichment of non-essential amino acids in cultured CHO cells

We improved carbon labeling of five non-essential amino acids, namely glutamate, aspartate, serine, glycine, and alanine, by up to 50% using the DCA medium. The NMR data indicate labeling of glutamine and/or asparagine that would be lost in our GC–MS derivatization and analysis scheme. We do not detect Cys or Arg in these cells, although they were detected in synthetic mixtures of amino acids. CHO cells are proline auxotrophs so this amino acid was not labeled. Consequently, the RN and DCA media improve isotope enrichment in at least 70% of the non-essential amino acids. Other studies also report significant labeling of these same amino acids from glucose (Deshpande et al. 2009). Enrichment with U-^{13}C-glutamate plus U-^{13}C-glucose yielded higher MPEs in these same amino acids, but we did not observe labeling in the other NEAAs. In most cases, the MPE was high considering that the medium contained unlabeled forms of each amino acid. The same amino acids were also enriched with similar MPEs in recombinant Thy1 (Table 1), indicating the efficient incorporation of these amino acids into recombinant proteins.

We investigated nitrogen metabolism in cells cultured under the DCA approach using ^{15}N-glutamate. Glutamate can be deaminated to α-ketoglutarate for metabolism via glutaminolysis pathway and the nitrogen can be used in transaminase reactions. Street et al. showed incorporation of 2-^{15}N-Gln-derived nitrogen into alanine and glutamate but not into free NH_4^+, suggesting an efficient transamination activity in CHO cells (Street et al. 1993). We observed ^{15}N enrichment from Glu into Asp, Ala, Gly, Ser and Lys. This is not too surprising as glutamate is the primary nitrogen donor in mammalian aminotransferases. On the other hand, the ^{15}N-MPEs were much lower than the ^{13}C-MPEs. In particular, it was surprising that the ^{15}N-MPE for Glu was very low (at roughly 30%) given the high enrichment of the starting material (which we confirmed). Interestingly, Street reported only ~50% ^{15}N enrichment of Glu from 2-^{15}N-Gln in the CHO K1 cells (Street et al. 1993). Together, these observations indicate the presence of a large, intracellular pool of (unlabeled) Glu in CHO cell and suggest that ^{15}N labeling may be more challenging than carbon labeling in mammalian cells.

Table 1 AT-MPE for five non-essential amino acids in cell extracts and in recombinant Thy1

Amino acid	Cell extract	Hydrolyzed Thy1
Ala	35	67
Gly	29	31
Ser	33	34
Asp	63	51
Glu	58	79

The observation of ^{15}N-labeled Lys was unanticipated. Although both nitrogens in Lys are derived from Glu, Lys is an essential amino acid. Lysine catabolism involves conversion to aminoadipate semialdehyde, catalyzed by lysine transaminase, with α-ketoglutarate serving as the N-acceptor. We speculate that this enzyme may operate in the reverse (amination) direction when cells are cultured under these conditions.

Gln, not Glu, is the nitrogen donor in the biosynthesis of N-acetyl glucosamine (GlcNAc), which is part of the core glycosylation found in N-linked glycoproteins. A previous study demonstrated incorporation of $^{15}NH_4^+$ into GlcNAc (Gawlitzek et al. 1999). The authors speculated that direct incorporation of ammonium may be an adaptation to the non-physiologically high NH_4^+ levels found in the CHO cell culture, but it is possible that $^{15}NH_4^+$ was first incorporated into 5-^{15}N-Gln and then into GlcNAc. The ^{1}H,^{15}N-HSQC of Thy1 grown in the presence of ^{15}N-Glu clearly shows resonances associated with the primary amides of Gln or Asn, but whether nitrogen from Glu are incorporated into carbohydrates found in glycoproteins requires further study.

Isotope labeling of recombinant proteins in CHO cells

The reduced nutrient medium formulation was developed for use in CHO cells, which express glutamine synthetase. The method works well for ^{13}C labeling but less well for ^{15}N labeling possibly due to label dilution from an unlabeled pool of Glu/Gln. ^{15}N-labeling may be increased by simply reducing (rather than replacing) Gln or by using a combination of ^{15}N-Glu and $^{15}N_2$-Gln. The requirement for expressing glutamine synthetase (GS) potentially limits the transfer of this method to other mammalian cells used for recombinant protein synthesis. However, BHK cells are readily adapted to glutamine-free growth media by inducing GS expression (Christie and Butler 1999). We found that HEK cells grown in RN and DCA media showed significantly improved cell viability and better attachment than HEK cells grown in SFM (not shown). Using the glutamine-synthetase amplifiable expression system (Bebbington and Hentschel 1987) to drive recombinant protein expression may extend the utility of this method to a wider variety of cells.

The structural consequences of post-translational modifications such as glycosylation have been poorly studied by NMR, in large part due to the difficulties of producing U-^{13}C,^{15}N-enriched proteins. The method outlined here does not produce such proteins, but suggests an approach to uniform labeling where isotope enriched essential amino acids are added to the culture medium while the non-essential amino acids are labeled using relatively low-cost precursors. Indeed, a recent paper (Deshpande et al. 2009)

demonstrates that CHO cell viability remains high in medium lacking all non-essential amino acids. The use of RN or DCA medium provides a novel method for producing recombinant proteins in CHO cells partially enriched with ^{13}C in a more cost-effective manner than addition of isotope enriched amino acids. Partial labeling can be particularly useful in biophysical studies of protein dynamics, protein–protein and ligand binding reactions, and as an aid to resonance assignments of high molecular weight proteins.

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