Development of a Human Cell-Free Expression System to Generate Stable-Isotope-Labeled Protein Standards for Quantitative Mass Spectrometry

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Overview

Purpose: To develop a human cell-free expression system for the production of stable-isotope-labeled (i.e. heavy) proteins.

Methods: HeLa cell lysates, supplemented with $^{13}C_6{}^{15}N_2$ L-Lysine and $^{13}C_6{}^{15}N_4$ L-arginine ,were used for *in vitro* translation (IVT) of recombinant fusion proteins. Labeled proteins were purified using affinity chromatography for mass spectrometry (MS) analysis.

Results: We developed a novel, human-cell-based IVT system which expresses heavy proteins with 90-97% stable isotope incorporation in less than eight hours.

Introduction

Stable-isotope-labeled peptides are routinely used as internal standards for the quantification of enzymatically-digested protein samples. Sable-isotope-labeled proteins are ideal for MS sample preparation standardization.[1] Traditional *in vivo* expression systems, such as ^{15}N-labeled *E. coli* or SILAC, have been used to express recombinant heavy proteins. However, these systems are limited in their expression of toxic or insoluble proteins, require two to three days for protein expression, and may have low yield of functional (i.e. properly folded) proteins. In addition, because *in vivo* systems use stable-isotope-labeled cell lines, all proteins in the cell are isotopically labeled leading to significantly higher waste and cost.

An alternative method to *in vivo* protein expression, *in vitro* translation (IVT), uses a cellular-extract system to transcribe DNA into mRNA, which is subsequently translated into protein. Most IVT systems utilize prokaryotic (e.g. bacteria) or non-human eukaryotic (e.g. rabbit reticulocyte) cell extracts.[2] However, these systems lack the components needed for proper folding and modification of human proteins, have lower expression yields, and inefficiently incorporate stable-isotope-labeled amino acids. In this study, we describe a novel human cell-free system[3,4] to express stable-isotope-labeled protein standards (Figure 1) as controls for sample-preparation loss, for digestion-efficiency determination, and as quantification standards.

Methods

Sample Preparation

Protein expression and purification: For each gene, full-length cDNAs were expressed as C-terminal fusion proteins using the Thermo Scientific 1-Step Heavy Protein IVT Kit. Depending on the C-terminal affinity tag, expressed proteins were purified using a Thermo Scientific Pierce GST Spin Purification Kit and/or a Thermo Scientific HisPur™ Cobalt Purification Kit. Purified protein samples were separated by SDS-PAGE and stained using Thermo Scientific Pierce GelCode Blue Stain Reagent. Gel slices containing each protein were destained, reduced, and alkylated before digestion to peptides using trypsin for 4-16 hours. After digestion, the peptides were desalted using Thermo Scientific C18 Stage tips and reconstituted with 0.1% TFA.

Expression of isotopically labeled proteins was performed in IVT reactions using a custom amino acid mix supplemented with the stable-isotope-labeled amino acids $^{13}C_6{}^{15}N_2$ L-Lysine and $^{13}C_6{}^{15}N_4$ L-arginine. All reactions were incubated at 30°C for 8–16 hours unless otherwise noted. Recombinant HIS-GFP protein fluorescence was measured using a GFP standard curve with a Tecan Safire™ fluorometer.

LC-MS/MS Analysis

A NanoLC-2D high-pressure liquid chromatograph (HPLC) with a Thermo Scientific PepMap C18 column (75 µm ID x 20 cm) was used to separate peptides using a 5–40% gradient (A: water, 0.1% formic acid; B: acetonitrile, 0.1% formic acid) at a flow rate of 300 nL/min for 40 min. A Thermo Scientific LTQ Orbitrap XL ETD mass spectrometer was used to detect peptides using a top-six experiment consisting of single-stage MS followed by acquisition of six MS/MS spectra with collision-induced dissociation (CID) to aid in protein identification.

Data Analysis

MS spectra were searched for matches with a custom human SWISSProt database using Thermo Scientific Proteome Discoverer software version 1.3, and the SEQUEST® search engine. Static modifications included carbamidomethyl with methionine oxidation. Lysine-8 and arginine-10 were used as dynamic modifications. SILAC ratios were based on the area under the curve (AUC) for each heavy and light peptide, and to determine stable-isotope incorporation.

FIGURE 1. Heavy recombinant protein expression, purification and MS analysis. A) IVT lysates were combined with the reaction mixture, vector DNA and stable-isotope-labeled amino acids to express recombinant proteins. B) Expressed proteins were then purified and digested into peptides for LC-MS analysis.

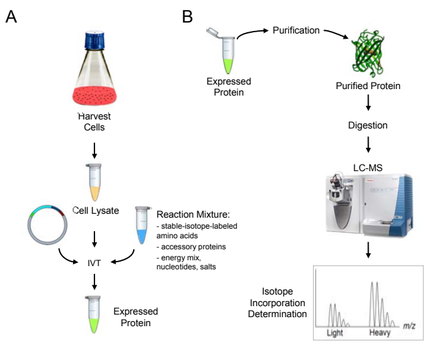


Figure 2. Heavy GFP protein expression. A) GFP was expressed for 20 hrs with increasing amounts of heavy arginine (Arg10) and lysine (Lys8), and analyzed using the workflow described in Figure 1. B) GFP expression over time showing corresponding heavy-isotope incorporation.

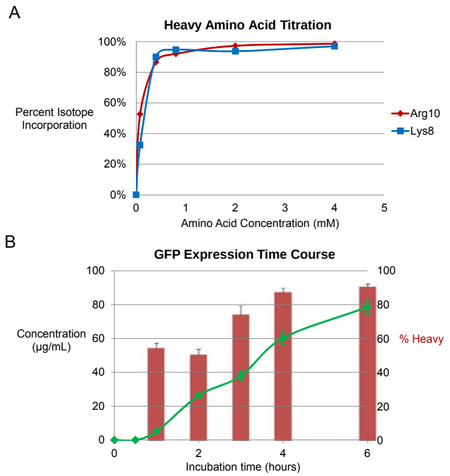
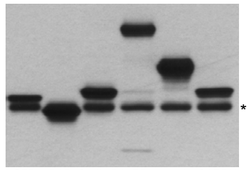


FIGURE 3. Expression of heavy mammalian proteins. A) Anti-GST Western blot of six different GST-fusion proteins expressed using human IVT extract (* indicates an anti-GST cross-reacting band in the lysate). B) Table showing isotope incorporation from peptides derived from the proteins shown in 3A.



Sample	MW	% Heavy
GFP	27 kDa	95%
BAD	19 kDa	92%
CyclinD1	36 kDa	97%
p53	53 kDa	91%
RB	110 kDa	96%
GAPDH	37 kDa	94%

FIGURE 4. MS spectra of peptides derived from proteins shown in 3A. A) MS spectra of stable-isotope-labeled GAPDH peptide AGAHLQGGAk. B) MS spectra of stable-isotope-labeled cyclin D1 peptide AYPDANLLNDr.

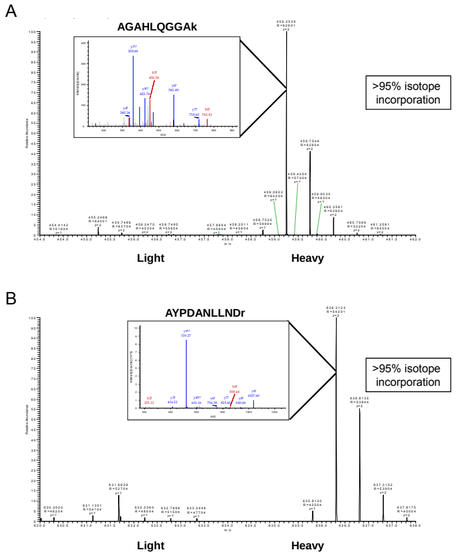
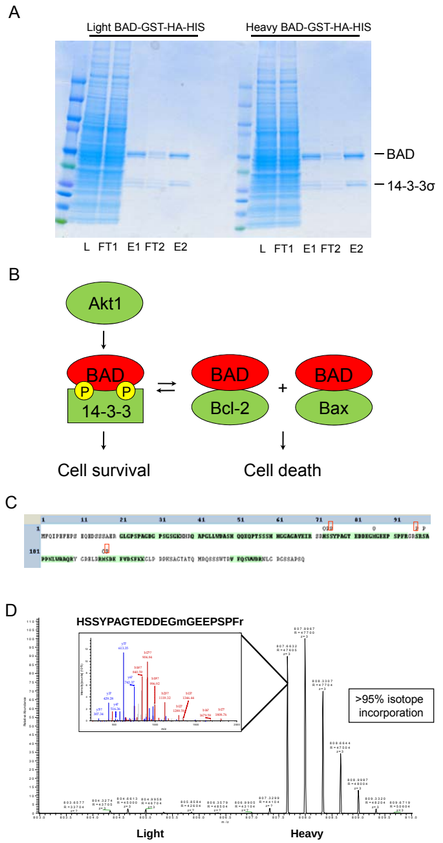


FIGURE 5. Heavy BAD protein-protein interaction. A) Coomassie-stained SDS-PAGE gel of recombinant light and heavy BAD-GST-HA-6xHIS purified from HeLa IVT lysates (L), using glutathione resin (E1) and cobalt resin (E2) tandem affinity. Flow troughs (FT) from each column are also indicated. B) Schematic of BAD phosphorylation and protein interactions during cell survival and cell death (i.e. apoptosis). C) BAD protein sequence coverage showing identified Akt1 consensus phosphorylation sites (red box). D) MS spectra of stable-isotope-labeled BAD peptide HSSYPAGTEDDEGmGEEPSPFr.



Results

Two different approaches for heavy-protein production were investigated. One used heavy SILAC-labeled cells to produce a heavy-labeled IVT extract. The other method used normal light IVT extracts supplemented with heavy amino acids. Although both methods successfully expressed GFP with stable-isotope incorporation of greater than 95%, the light lysates had a significantly higher level of protein expression and lower cost of production (Figure 2A). Titration and time-course experiments using the light lysate with heavy amino acids showed that amino acid concentrations greater than or equal to 1 mM and incubation times longer than four hours were necessary for optimal protein expression and stable-isotope incorporation (Figure 2A and 2B).

In order to validate the use of this human heavy IVT system for production of human-based proteins, six additional recombinant proteins were expressed and purified using GST or 6xHIS affinity purification. Although all proteins were expressed as indicated by a Western blot (3A), only four of the six proteins were recovered after purification and sample preparation with high yield. As determined by our MS analysis of heavy and light peptides, all expressed proteins had stable-isotope incorporation equal to or greater than 90% (Figure 3B and Figure 4).

Ideal protein standards are identical to their endogenous counterparts. Expression of recombinant proteins in human cell-free extract systems has been shown to aid in proper protein folding and post-translational modification.[2] During the purification of one mammalian protein, BAD, we observed co-purification of light 14-3-3σ with the heavy protein (Figure 5A). This protein-protein interaction is known to be mediated by 14-3-3 binding of serine/threonine phosphorylation motifs (Figure 5B).[5] MS analysis of the IVT-expressed protein identified three of four Akt consensus phosphorylation sites (Figure 6C). Overall, these results indicate that recombinant BAD expressed using this human cell-free expression system has functional protein modifications and interactions.

Conclusions

- Six different stable-isotope-labeled proteins were produced using a modified non-SILAC human IVT system.
- Isotope incorporation efficiency of greater than 95% was observed for IVT reactions containing stable-isotope amino acids at concentrations greater than or equal to 50 mM and incubated for longer than four hours.
- Co-purification of heavy BAD protein with light 14-3-3 proteins suggests proper protein folding and post-translational modification of *in vitro* expressed protein.

References

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