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Stable isotope labeling by amino acids in cell culture-based liquid chromatography–mass spectrometry assay to measure microtubule dynamics in neuronal cell cultures

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ABSTRACT

Microtubules (MTs) are highly dynamic polymers composed of α- and β-tubulin heterodimers. Dysregulation of MT dynamics in neurons may be a contributing factor in the progression of various neurodegenerative diseases. We developed a stable isotope labeling by amino acids in cell culture (SILAC)-based liquid chromatography–mass spectrometry (LC–MS) method to measure the fraction of [$^{13}C_6$]leucine-labeled α-tubulin-derived surrogate peptides. Using this approach, we measured the time course of incorporation of [$^{13}C_6$]leucine label into the MT and dimer pools isolated from cycling cells and rat primary hippocampal neurons. We found that the MT pool is in rapid equilibrium with the dimer pool in the cycling cells, consistent with rapid MT polymerization/depolymerization during cell proliferation. Conversely, in neurons, we found that labeling of the MT pool was rapid, whereas the dimer pool was delayed. These results suggest that newly synthesized α-tubulin is first incorporated into MTs or complexes that co-sediment with MTs and that appearance of labeled α-tubulin in the dimer pool may be a consequence of MT depolymerization or breakdown. Our results demonstrate that a SILAC-based approach can be used to measure MT dynamics and may have utility for exploring MT dysregulation in various models of neurodegenerative disease.

Microtubules (MTs)[3] are highly dynamic polymers composed of α- and β-tubulin heterodimers that play a critical role in the regulation of axonal transport and synaptic function in neurons [1]. Dysregulation of MT dynamics may contribute to the deficits in axonal transport observed in neurodegenerative diseases such as Alzheimer's disease [2,3]. Robust and quantitative methods to accurately measure MT dynamics in neurodegenerative disease models are needed to further explore the link between regulation of MT dynamics and the disease process.

Efforts to measure MT dynamics in cell culture systems have relied primarily on imaging-based approaches [4,5]. Although informative, these methods are potentially less suited to support a drug screening effort given the need for relatively high throughput and quantitative assays. Recently, pulse labeling with D_2O has been used to monitor incorporation of α- and β-tubulin heterodimers into newly synthesized MT polymers as a measure of MT dynamics in cell culture and in vivo models [6–8]. Although these results provide proof of concept for the idea that pulse labeling can be used to monitor MT dynamics, a major limitation with the deuterium labeling approach is the requirement for relatively pure preparations of MT polymers and dimers for analysis.

In this study, we developed a liquid chromatography–mass spectrometry (LC–MS) assay based on stable isotope labeling by amino acids in cell culture (SILAC) methodology [9–11]. SILAC is based on the metabolic incorporation of labeled amino acids into target proteins that then can be detected by MS as a mass shift in a proteolytically derived surrogate peptide [12,13]. The ratio of the labeled to unlabeled peak intensities can be used to monitor surrogate labeling independent of absolute abundance. The identity of the surrogate peptides can be verified by LC–MS/MS (tandem mass spectrometry), thereby circumventing the need for pure samples. Data presented here demonstrate the utility of this

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[3] *Abbreviations used:* MT, microtubule; LC–MS, liquid chromatography–mass spectrometry; SILAC, stable isotope labeling by amino acids in cell culture; MS/MS, tandem mass spectrometry; DIV, days in vitro; HPLC, high-performance liquid chromatography; CE, collision energy; CV, coefficient of variation.

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SILAC-based LC–MS technique to measure MT dynamics in cycling cells and primary rat hippocampal neurons.

Materials and methods

Sample preparation

Primary rat brain tissue was isolated from E18 embryonic rats following animal care and use committee approved guidelines. Neurons were isolated from hippocampus using the Papain Dissociation System (Worthington, Lakewood, NJ, USA). Cells were plated at 1×10^6 cells/well on polyornithine-coated 6-well plates in Neurobasal medium containing B27 supplement with 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine to select for neuronal cells. The majority (>90%) of cells in these cultures consist of neurons as determined by immunostaining for NeuN (a marker for neurons). To generate cultures of cycling cells (astrocytes), cells isolated from the hippocampal tissue were replated in Dulbecco's modified Eagle's medium (DMEM)/F12 (50:50) with 100 U/ml penicillin, 100 μg/ml streptomycin, 0.5% GlutaMAX, and 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA). This protocol allows astrocyte propagation such that more than 90% of the cells are astrocytes based on immunostaining for GFAP (a marker for astrocytes). Medium changes were performed on the first day in vitro (DIV) and on 7 DIV. At 11 DIV, the stable isotope [$^{13}C_6$]leucine (Cambridge Isotope Labs, Cambridge, MA, USA), referred to as *L, was added to the cells at a final concentration of 105 μg/ml, representing 50% of total leucine (L) present in the medium. Cells were incubated for up to 7 days/168 h (18 DIV) after the addition of label and samples taken at various timepoints. Replicate wells (n = 3/timepoint) were processed individually. Three separate tissue preparations were used to generate experimental replicates.

MT isolation was performed essentially as described previously [6] with the modifications outlined below. Cells were lysed in 300 μl/well in MT stabilization buffer (80 mM K-Pipes, 1 mM ethyleneglycoltetraacetic acid [EGTA], and 1 mM $MgCl_2$, pH adjusted to 6.8 with 1 M KOH) containing 20% sucrose. Cell lysates were centrifuged at 5000g for 5 min at room temperature to pellet the cell nuclei. Post-nuclear supernatants were then centrifuged at 190,000g for 35 min at 25 °C. Supernatants were collected and labeled as the dimer fraction. α-Tubulin was purified from the dimer fraction by immunoprecipitating overnight at 4 °C with a monoclonal antibody against α-tubulin (Sigma, St. Louis, MO, USA) directly conjugated to beads (AminoLink Plus resin, Thermo Scientific, Rockford, IL, USA). This immunoprecipitation step was needed to further enrich and concentrate the tubulin present in the dimer fraction. The α-tubulin was eluted with 30 μl of 6 M urea containing 50 mM Tris–HCl (pH 8.5). The eluted samples were stored at −20 °C until enzymatic digestion. The polymeric tubulin pellets were resuspended in 30 μl of Tris–HCl (50 mM, pH 8.5) containing 6 M urea. Samples were incubated on ice for 2 h and then stored at −80 °C until enzymatic digestion. For digestion, the samples were sonicated for 30 min and chilled for 60 min to disrupt the MTs. Then, 1.5 μl of dithiothreitol (DTT; 100 mM stock, final concentration of 5 mM) was added and the samples were incubated for 30 min at 37 °C. Next, 1 μl of iodoacetamide (200 mM stock, final concentration of 6 mM) was added and the samples were incubated for 30 min at room temperature in the dark. Lastly, 2 μl of Trypsin/Lys-C Mix (0.2 μg/μl stock, final concentration of 12 ng/μl) (Promega, Madison, WI, USA) was added to the samples, followed by incubation for 3 h at 37 °C. The samples were then further diluted with 150 μl of Tris–HCl (50 mM, pH 8.5) and incubated overnight at 37 °C. The reaction was quenched with 0.5 μl of formic acid and cooled to 4 °C. The samples were evaporated to dryness by speed vacuum and resuspended in 40 μl of water/acetonitrile (95:5, v/v). The samples were stored at −80 °C until analysis by LC–MS.

Analytical details

Unless otherwise noted, high-performance liquid chromatography (HPLC)-grade solvents and analytical-grade chemicals were used.

The HPLC–MS system consisted of a Leap Technologies CTC HTS PAL autosampler (Carrboro, NC, USA), an Agilent Technologies 1100 Capillary LC pump (Wilmington, DE, USA), and a Thermo Scientific hybrid linear ion trap LTQ XL Fourier transform Orbitrap mass spectrometer (San Jose, CA, USA). For LC–MS analysis, 10 μl of sample was injected onto a Phenomenex Jupiter Proteo column (50 × 1 mm, 4 μm, 90 Å; Torrance, CA, USA). The mobile phases were water/acetonitrile (98:2, v/v) (mobile phase A) and acetonitrile/water (98:2, v/v) (mobile phase B). The water in each of the solvent mixtures contained 0.1% formic acid. The LC was performed at a flow rate of 70 μl/min with the eluant diverted to waste for the first 3 min. After injection, the mobile phase composition was held at 5% B for 0.5 min and a linear gradient was then executed to 25% B over 10 min, followed by another linear gradient to 80% B over 2 min. The mobile phase composition was held at 80% B for 0.5 min, followed by a step gradient to initial conditions and a 6-min reequilibration period. Total analysis time was 19 min.

The mass spectrometer was operated in positive ion mode with the following ionization source conditions: spray voltage 5 kV, capillary voltage 48 V, tube lens voltage 105 V, capillary temperature 300 °C, sheath gas flow 40 arbs, auxiliary gas flow 5 arbs, and sweep gas flow 5 arbs. High-resolution accurate mass qualitative MS and MS/MS spectra were acquired in profile mode on the Orbitrap instrument with 30,000 resolution (full width at half height maximum, FWHM) and data-dependent mode with a 2-Da isolation window and 35% collision energy (CE). The data were used to determine the amino acid residue sequence of the peptidic analytes. Low-resolution quantitative data were acquired in centroid mode on the ion trap instrument with 1 microscan, 500 ms ion time, 2-Da isolation window, and 35% CE. The transitions of the most abundant signal in the doubly charged/protonated isotopic envelope of the precursor ion, $[M+2H]^{2+}$, to the most abundant singly charged/protonated product ion were selectively monitored (selected reaction monitoring, SRM): for L-F16 at m/z 913.1 to 1150.4 (m/z 913.1 → 1150.4 ± 0.5 Da), for *L-F16 at m/z 916.1 to 1156.4 (m/z 916.1 → 1156.4 ± 0.5 Da), for L-F18 at m/z 444.4 to 625.2 (m/z 444.4 → 625.2 ± 2.5 Da), and for *L-F18 at m/z 447.4 to 631.2 (m/z 447.4 → 631.2 ± 2.5 Da).

Sample injection carryover was minimized with an injection port cleaning procedure based on the Clean LC macro as part of the Leap Technologies autosampler operating software, Cycle Composer [14]. Briefly, the injection loop is taken offline during an LC run and the loop, injector needle, and injector syringe barrel are then washed consecutively with 10 mM ethylenediaminetetraacetic acid (EDTA) in 2% acetonitrile and 10 mM ammonium hydroxide in acetonitrile. The macro is included in the sample sequence so that cleaning occurs after each injection.

Data were processed using the Thermo Scientific MS software Xcalibur and the auxiliary tool FT Programs–Protein Calculator. Protein databases were searched with Peaks Studio 5.2 software (Bioinformatics Solutions, Waterloo, Ontario, Canada). A tubulin standard (>99% isolated from bovine brain) purchased from Cytoskeleton (Denver, CO, USA) was used to construct a standard curve to evaluate linearity and sensitivity of the MS peaks. Data analysis was performed using GraphPad Prism software (San Diego, CA, USA).