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Quantitative comparison of CrkL-SH3 binding proteins from embryonic murine brain and liver: Implications for developmental signaling and the quantification of protein species variants in bottom-up proteomics

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ABSTRACT

A major aim of proteomics is to comprehensively identify and quantify all protein species variants from a given biological source. However, in spite of its tremendous utility, bottom-up proteomic strategies can do little to provide true quantification of distinct whole protein species variants given its reliance on proteolysis. This is particularly true when molecular size information is lost as in gel-free proteomics. Crk and CrkL comprise a family of adaptor proteins that couple upstream phosphotyrosine signals to downstream effectors by virtue of their SH2 and SH3 domains respectively. Here we compare the identification and quantification of CrkL-SH3 binding partners between embryonic murine brain and liver. We also uncover and quantify tissue-specific variants in CrkL-SH3 binding proteins.

1. Introduction

The proto-oncogene *c-Crk* (CT10 regulator of kinase) and its relative *CrkL* (Crk-like) are cellular homologues of the viral oncogene *v-Crk* carried by the avian sarcoma virus CT10 [1,2]. Although devoid of enzymatic activity, the Crk and CrkL proteins facilitate signal transduction by linking proteins containing phosphorylated tyrosine residues to downstream effectors. Crk and CrkL perform this role by virtue of their simple dual domain structure consisting of an amino-terminal Src Homology 2 (SH2) domain and either one or two carboxyl-terminal SH3 domains. Their SH2 domains bind to proteins with phosphorylated tyrosine in YxxP motifs and their SH3 domain binds to proteins harboring PxxPxK motifs [2,3]. The substrates of many tyrosine kinases recruit Crk and CrkL and thereby regulate an array of signaling pathways [2–4]. Crk and CrkL play overlapping roles and are essential for proper development in the mouse, most evident by the early lethal phenotype that results in compound *c-Crk* and *Crkl* mutants [5–7]. However, genetic disruption of only one family member can still have important effects [5–7]. Previously we found that Crk and CrkL were recruited to tyrosine phosphorylated Disabled-1 (Dab1), a critical scaffold regulating Reelin signaling in mammalian brain development [8]. The essential nature of Crk and CrkL in Reelin signaling was given genetic support using Cre-Lox-mediated compound disruption of their encoded genes conditionally in the developing nervous system [7]. The recruitment of Crk and CrkL to phoshpho-tyrosyl Dab1 co-translocates their SH3-binding proteins including the Rap-GEF C3G (CrkL SH3-binding Guanine Nucleotide-Releasing Protein) [8]. Furthermore, we recently identified several additional CrkL-SH3 binding partners from embryonic murine brain [3]. Given Reelin's ability to cluster several receptors [9,10] this leads to the potential of multiple complex intracellular signaling assemblages proximal to Reelin receptors.

In order to determine if the CrkL-SH3 binding proteins we identified in embryonic brain were distinct from CrkL-SH3 binding partners in other tissue types, we used quantitative mass spectrometry to compare CrkL-SH3 binding proteins from embryonic murine brain and liver lysates. CrkL-SH3 binding proteins were eluted and subjected to SDS-PAGE. Protein regions from the entire gel were subjected to in-gel tryptic digestion. Extracted peptides were subjected to labeling by reductive amination using reagents with differential masses based on stable isotopes. Following liquid chromatography tandem mass spectrometry (LC–MS/MS), a total of 40 CrkL-SH3 binding proteins common to two biological replicates were quantified. 30 were enriched in the brain pulldowns while three were enriched in the liver pulldowns. Three proteins showed no enrichment in the pulldowns while four of the proteins showed the striking behavior of variant-specific enrichment, at least when considering differences in molecular weight as a proxy for protein

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species variants. This concept is further discussed with particular consideration paid to signatures of protein species variants in quantitative bottom-up proteomic workflows.

2. Material and methods

2.1. Mice, plasmids and antibodies

Timed pregnant CD-1 mice were obtained from Charles River Laboratories, Canada (Saint-Constant, Québec, Canada) and treated according to an institutionally-approved IACUC protocol (#10-068). Mice were euthanized after a brief isoflurane administration when embryos were at embryonic day 16.5 (E16.5). The embryonic brains and livers were carefully dissected and tissue was lysed as described below. The bacterial expression plasmid encoding GST (pGEX-4T-1) was from Stratagene/Agilent Technologies (Santa Clara, CA, USA) and the plasmid encoding GST-CrkL-SH3 was a gift of Akira Imamoto (University of Chicago, USA) [5]. The following antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA): α-C3G (H-300), α-DDEF2/ASAP2 (H-300), α-N-WASP (H-100), and α-PEX13 (H-300).

2.2. Affinity chromatography and GST fusion protein pulldown assays

E16.5 murine whole brain and liver extracts were generated by dounce homogenization in ice-cold brain complex lysis buffer (BCLB: 25 mM Tris pH 7.2, 137 mM NaCl, 10% glycerol, 1% Igepal, 25 mM NaF, 10 mM $Na_4P_2O_7$, 1 mM Na_3VO_4, 1 mM PMSF, 10 μg/ml leupeptin, and 10 μg/ml pepstatin A). The supernatants were collected after lysates were centrifuged at 4 °C for 20 min at 16,000 ×*g*. Three milliliters of each supernatant, corresponding to 5 mg of protein was pre-cleared by rocking with 100 μl of a 50% slurry of BCLB-washed glutathione agarose resin (G-Biosciences, Maryland Heights, MO, USA) for 1 h at 4 °C. After centrifugation for 10 min at 16,000 ×*g* the supernatants were collected. Each supernatant was then similarly pre-cleared by rocking for 3 h at 4 °C with 70 μl of a 50% slurry (in BCLB) of glutathione agarose bound to 35 μg of GST. The resins were collected by brief centrifugation and the supernatants were retained. The GST resin was then washed three times with BCLB, drained and proteins were eluted from the resin with protein sample buffer (125 mM Tris pH 6.8, 2% SDS, 5% β-mercaptoethanol, 7.5% glycerol) at 95 °C for 5 min. The eluates were stored at −20 °C for later use. The GST pre-cleared supernatants were rocked overnight at 4 °C, each with 70 μl of a 50% glutathione agarose slurry (in BCLB) bound to 35 μg GST-CrkL-SH3. Resins and their associated proteins were collected by centrifugation and washed three times with BCLB and drained. Protein sample buffer was added and samples, including the GST pre-cleared resins, were heated to 95 °C for 5 min. Eluted proteins were separated using a 7.5% (37.5:1 acrylamide:bis-acrylamide) SDS-PAGE gel and then stained with Coomassie blue.

2.3. In-gel reduction, alkylation and tryptic digestion

The lanes of the Coomassie-stained gel from the brain and liver pulldowns were each cut into 15 equal regions above the CrkL-SH3 band. The gel pieces were diced into 1 mm cubes, washed with 1 ml of HPLC-grade water and incubated in 1 ml of destain solution (50 mM ammonium bicarbonate and 50% acetonitrile (MeCN)) for 30 min at 37 °C. For full removal of the stain, destaining was repeated once again and then gel slices were subjected to dehydration by adding 100% MeCN for 10 min. The MeCN was then removed and the gel slices were dried. The proteins in the gel pieces were then reduced in 10 mM of DTT in 50 mM of ammonium bicarbonate and by incubation at 56 °C for 20 min. The samples were allowed to cool down to room temperature and the overlay solution was drained. The gel pieces were then dehydrated with 100% MeCN, rehydrated with water and then dehydrated again with MeCN. Proteins were next alkylated in 50 mM of iodoacetamide in 50 mM of ammonium bicarbonate while incubating in the dark at room temperature for 1 h. After removing the samples from the dark, the alkylation solution was discarded and the gel pieces were washed with 1 ml of HPLC-grade water and then with 500 μl of destain solution. The gel pieces were then dehydrated using 100% MeCN, allowing 5 min for each step. The washing was repeated twice and the gel pieces were then dehydrated using 100% MeCN and then dried in a speed vacuum for 15 min. Proteins were cut into peptides using sequencing grade modified trypsin (Promega, Madison, WI, USA) at a concentration of 6 ng/μl in 50 mm of ammonium bicarbonate at 37 °C overnight. The in-gel digests were centrifuged for 5 min at 13,000 ×*g* and the supernatant was transferred to a 0.6 ml tube. Extraction solution (50% MeCN, 2.5% formic acid (FA)) was then added to the gel pieces and they were centrifuged at 13,000 ×*g* for 15 min. Extractions were combined with appropriate digests and dried in a speed vacuum.

2.4. Peptide labeling, liquid chromatography tandem mass spectrometry (LC–MS/MS), and data analysis

The labeling of tryptic peptides with mass tags was done using reductive amination (dimethylation) chemistry [11] in which peptides are modified using light (^1H) or heavy (^2H) formaldehyde and sodium cyanoborohydride. Heavy reagents were purchased from Cambridge Isotopes Laboratories, Tewksbury, MA, USA and light reagents were from Sigma (St. Louis, MO, USA). Embryonic brain and liver peptides derived from bound proteins in the CrkL-SH3 pulldowns were labeled using light and heavy reagents respectively. Peptides collected from each gel region were resuspended in 40 μl of 1 M HEPES, pH 7.5. 4 μl of light labeling reagents (4% formaldehyde, 600 mM sodium cyanoborohydride) were added to each tube containing brain peptides and 4 μl of heavy labeling reagents (4% $^2H_2$-formaldehyde, 600 mM sodium cyanoborodeuteride) were added to each tube containing liver peptides. The reactions proceeded for 10 min and then the same volumes of labeling reagents were added again to push the reaction to completion. The reaction was allowed to continue for 10 additional minutes and then was quenched by adding 50 μl of 10% trifluoroacetic acid (TFA). Both light and heavy reactions were allowed to sit at room temperature for 1 h and then mixed together carefully so that reactions representing each gel region from the brain samples were mixed with reactions representing the corresponding regions of the liver samples. Mixed peptides were then subjected to desalting using C18 spin columns (Thermo Electron, San Jose, CA, USA). Finally, the peptides eluted from the spin columns were dried in a speedvac and resuspended in 8 μl of 2.5% FA, 2.5% MeCN. 4 μl of the sample was loaded using a Micro AS autosampler (Thermo Electron) onto a microcapillary column of 100 μm inner diameter packed with 12 cm of reversed-phase Magic C18 packing material (5 μm, 200 Å; Michrom Bioresources, Inc., Auburn, CA, USA). After a 14.5 min isocratic loading in 2.5% MeCN, 0.15% FA (Solvent A) peptides were eluted using a 5%–35% gradient of Solvent B (98.85% MeCN, 0.15% FA) over 30 min and electrosprayed into a linear ion trap-orbitrap (LTQ-Orbitrap) mass spectrometer (Thermo Electron). The precursor scan was followed by ten collision-induced dissociation (CID) tandem mass spectra for the top 10 most abundant ions. Dynamic exclusion was enabled with a repeat count of three and a repeat cycle of 180 s. Lock mass was enabled and set to calibrate on the mass of a polydimethylcyclosiloxane ion ([(Si(CH3)2O)5 + H+]+, m/z = 371.10120). Tandem mass spectra were searched against a concatenated forward and reverse [12] mouse NCI protein database using SEQUEST (version 27 revision 12) requiring: fully tryptic peptides, a mass addition of 57.02146 Da for carbamidomethyl adduction on cysteines, a mass addition of 28.03130 Da on amino termini and lysines; and allowing for: a precursor mass tolerance of 40 PPM, a differential mass addition of 15.99491 Da on methionine, and a differential mass addition of 6.03766 Da on amino termini and lysines. SEQUEST matches in the first position were then filtered by XCorr scores of 1.0, 1.5, 1.8 and 2.0 for the charge states of plus one, two, three and four respectively. A ΔCn2 score of 0.2 was also required for each peptide. Protein matches