Affinity Purification Of Ubiquitinated Proteins From Rat Brain Tissue

George E. Craft[1], Kathryn Stone[2] and Angus C. Nairn[1]
[1]Division of Molecular Psychiatry, Yale University, New Haven, CT, USA
[2]W.M. Keck Foundation, Keck MS and Proteomics, Yale University, New Haven, CT, USA

639.20/D4

INTRODUCTION

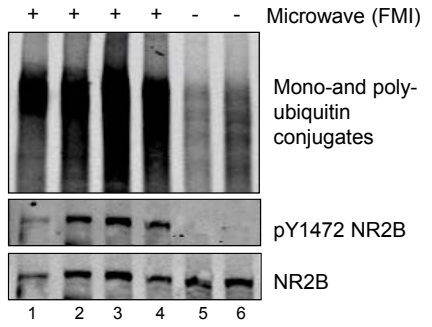
- Post-translational modifications allow dynamic regulation of proteins in the nerve terminal that can respond quickly to physiological and non-physiological stimuli
- Protein ubiquitination plays an important role in many aspects of neuronal function including regulation of endocytosis, signaling, gene expression and protein degradation by the proteasome
- Protein ubiquitination is a dynamic process in the cell and is intricately regulated
- Ubiquitin binding domains (UBD's) are found in many proteins and have differing specificity for mono- and poly ubiquitin
- Rapid heating of rat and mouse brain tissue using Focused microwave irradiation (FMI) inactivates protein phosphatase activity (O'Callaghan *et al*, 2004)
- Proteomic based strategies in yeast (Peng, J., *et al* 2003), plant, HEK cells, heat brain and tissue all been used to purify ubiquitinated proteins
- We are interested in purifying ubiquitinated proteins from brain tissue with the aim to purify ubiquitinated proteins after exposure to drugs of abuse

METHODS

Rats were sacrificed either by rapid decapitation or by Focused Microwave Irradiation (FMI) according to Yale University IACUC approved methods. Biochemical sub-cellular fractionation of rat brain tissue was performed as previously described (Hallett *et al* 2008). Settings for the FMI instrument were optimized for complete inactivation of brain enzymes. Proteins in each fraction were incubated with Ubiquitin Binding Domains (UBD's) to purify ubiquitinated proteins. GST-S5a, GST-Ataxin-3-UIM, Dsk2-UBA (Biomol) and Tubes 1 (LifeSciences) were used in the study. The bound proteins were washed and eluted using SDS buffer and analyzed by SDS-PAGE. Proteins were transferred to nitrocellulose membrane for western blotting, incubated with the indicated primary antibodies. Fluorescent secondary antibodies were used to detected using the LI-COR imaging system. The remainder of the eluted protein was analyzed by SDS-PAGE, proteins detected using Coomassie blue protein gel stain and gel bands cut out and subjected to trypsin digestion. Tryptic peptides were separated by the nanoACQUITY Ultra Performance LC and analyzed on the Thermo linear Ion trap (LTQ)- Orbitrap XL mass spectrometer (Thermo-Electron Corp) at the Keck laboratories. The MS data was processed and files searched against the rat database using the MASCOT server. The search was performed using the search parameters choosing trypsin with 2 miss-cleavages with variable modifications; Propionamide (C), Oxidation (M) and GlyGly (K).

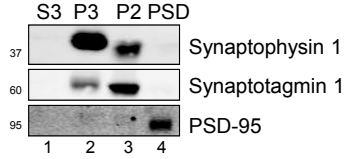
RESULTS

1. FMI inactivates protein phosphatase and activity



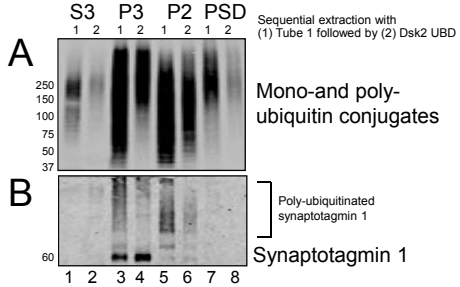
Rats sacrificed by microwave (lanes 1-4) were compared to fresh dissection (lanes 5-6). Total mono-and poly-ubiquitin (FK2 antibody) signal was higher in FMI samples compared to fresh tissue, highlighting post-mortem DUB activity

2. Biochemical fractionation of rat brain tissue



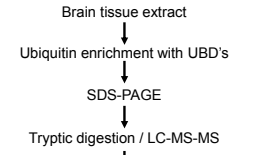
Fresh brain tissue was homogenized and fractioned into S3 (cytosol), P3 (light membranes), P2 (synaptosomal) and PSD (Post-Synaptic Density). Synaptophysin and synaptotagmin migrated higher in the P3 (light membrane) fraction compared to the P2 synaptosomal fraction. A higher proportion of synaptophysin was in the P3 fraction

3. Enrichment of mono- and poly-ubiquitinated proteins from brain tissue



Sequential enrichment of ubiquitinated proteins using the fractionated extracts (S3, P3, P2 and PSD) using the Tube 1 UBD (lanes 1, 3, 5, 7) followed by a second extraction of the brain extract using the Dsk2 UBD (lanes 2, 4, 6, 8). **(A)** Total mono- and poly ubiquitin was detected by western blotting using the FK2 antibody. **(B)** Synaptotagmin 1 antibody detected a signal at 60 kDa in the P3 fractions (lanes 3 - 4) and low signal in the P2 lanes (lanes 5 - 6). This matches the higher band observed in the total lysate (Fig. 2, lane 2). A Synaptotagmin smear was observed in both the P2 and P3 fractions indicating poly-ubiquitination.

4. Identification of ubiquitinated proteins by mass spectrometry



5. Summary of ubiquitinated proteins identified from brain tissue

Cytoskeletal: actin, α/β-tubulins, neurofilaments medium and large, myosin6, GFAP, MAP1a/1b, MAP2, MAP6, α-adducin, septins-4/7/11

Translation: Eef1g, Eef1a1/2, Eef2, Eef1d, Eef1g

Chaperones: Hspa1(HSP-70), Hspaa1(HSP-90), HSP-84, Hspa2, Hspa4, Hsp5, Hspa9

Proteasomal subunits: Psmd1 26S non-ATPase subunit 1, Psmd1-p112 subunit, Psmc1-5 26S regulatory subunits, Psmd2, Psmd3, E2 ubiquitin conjugating enzyme, E3 ubiquitin ligases, UCHL1, UCHL5

Metabolism:, Glucose-6-phosphate isomerase, Pyruvate kinase, guanine deaminase, enolase

Kinases: CaMKII-α/β, PKA, PKC, CaM kinase, MARK2, creatine kinase, phosphoglycerate kinase, Pfkm 6-phosphofructokinase

Phosphatases: PP2A-catalytic subunit, PP2A-regulatory subunit, PP2B, receptor-type tyrosine-protein phosphatase zeta

Synaptic proteins: synaptotagmin, synaptobrevin, syntaxin, SNAP25, epsin, synapsin I/II, dynamin, Nsf vesicle-fusing ATPase , Homer, SV2A, spinophilin, clathrin heavy chain, AP2-α/β/μ, Shank1/3, AKAP5, PSD-95, NCAM, 14-3-3, NR2B, GluR2, Rabphilin3A, small GTPases, rab3A, Rab1, GAT-1,

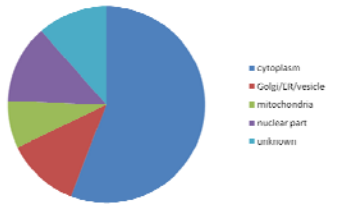
Mitochondrial proteins: ATP synthase subunit β, Glud1, Hexokinase1/2

Nuclear proteins: Histones H1, H2A, H2B and H3, nucleolin like protein, HDAD6

A total of 548 proteins were identified in this study.

Ubiquitinated peptides were identified for a number of the proteins. Manual validation of ubiquitinated peptides is still in progress.

6. Cellular location of ubiquitinated proteins



Bioinformatics was used to analyze the list of ubiquitinated protein identifications according to cellular location.

SUMMARY

- Ubiquitinated proteins were purified from rat brain tissue
- FMI is an effective method to inactivate DUBs in brain tissue, reflecting the very active level of post-mortem activity
- Over 548 ubiquitinated proteins identified from brain tissue by mass spectrometry
- Using the characteristic double glycine (-GG) modification (114 Da mass shift) resulting from tryptic digestion, specific sites of ubiquitination were identified
- Challenge remains to capture ubiquitinated proteins that are rapidly degraded by the proteasome
- Ongoing studies examining activity-dependant changes in protein ubiquitination after exposure to drugs of abuse

REFERENCES

Hallet, P.J., Collins, T.L., Standaert, D.G., Dunah, A.W. (2008) Biochemical fractionation of brain tissue for studies of receptor distribution and trafficking. Current Protocols in Neuroscience. 1.16.1-16.

O'Callaghan, James P., Krishnan Sriram. (2004) Focused microwave irradiation of the brain preserves in vivo protein phosphorylation: comparison with other methods of sacrifice and analysis of multiple phosphoprotein. Journal of Neuroscience methods. 135: p159-168.

Peng,J., Schwartz,D., Elias,J.E., Thoreen,C.C., Cheng,D., Marsischky,G., Roelofs,J., Finley,D., Gygi, S.P.(2003). A proteomics approach to understanding protein ubiquitination. Nat. Biotechnol. 21:p921-926.

SUPPORT

This project was supported by the Dept. of the Army USAMRAA W23RYX-9049-N610