Affinity purification of ubiquitinated proteins and peptides from rat brain extracts

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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.

❖ Ubiquitin binding domains (UBD's) are found in many proteins and have differing specificity for mono- and poly ubiquitin

❖ After trypsin digestion, ubiquitinated peptides retain the two C-terminal glycine residues attached by a peptide bond. A peptide mass increase of 114 Da was used to assign specific-sites of protein

❖ Ubiquitin Branch Motif (K-ε-GG) antibody can be used to selectively enrich for ubiquitinated peptides after trypsin digestion (Kim, W., et al, 2011 and Lee, K.A., et al, 2011)

❖ 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). Primary cortical cultures were prepared from cortices at E18 and cultured using standard methods for DIV14-21.

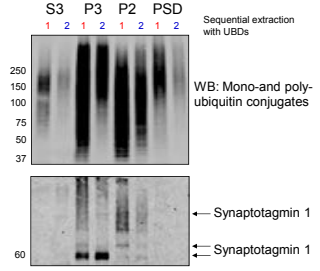
Enrichment of ubiquitinated proteins from protein extracts. Protein extracts were incubated with Ubiquitin Binding Domains (UBD's) to purify ubiquitinated proteins. GST-S5a, GST-Ataxin-3-UIM, Dsk2-UBA (Biomol),Tubes 1 and Tubes 2 (LifeSciences) were used in the study. The bound proteins were washed and eluted using SDS buffer and analyzed by SDS-PAGE. Proteins were transferred to nitrocellulose membrane for western blotting, incubated with primary antibodies and scanned using the LICOR 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 and LC-MSMS.

Enrichment of ubiquitinated peptides from protein extracts. Protein Extracts were digested with Lys-C (3h, RT), diluted and then digested with trypsin (16h, 37° C). Peptides were purified using a Sep-Pak cartridge and freeze-dried overnight. The Ubiquitin Branch Motif (K-ε-GG) Immunoaffinity Beads (Cell Signaling Technology cs#1990) was used to immunoprecipitate ubiquitinated peptides from the peptide mixture. Beads were washed and bound peptides eluted using 0.15% TFA.

Mass spectrometry. 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 SWISSPROT (rat) database using the MASCOT server. The search was performed using the search parameters choosing trypsin with 3 miss-cleavages with variable modifications Propionamide (C), Oxidation (M), Deamidated (NQ) and GlyGly (K). Ubiquitinated petides were identified by mass spextrometry by 114 Da mass increase (GlyGly (K) left over after trypsin digestion.

RESULTS

1. Enrichment of ubiquitinated proteins using UBD's from rat brain tissue



Fresh brain tissue was homogenized and fractioned into S3 (cytosol), P3 (light membranes), P2 (synaptosomal) and PSD (Post-synaptic Density).

(A) Ubiquitinated proteins were enriched using Tubes 1 and Tubes 2 UBD's and mono-and poly-ubiquitin proteins detected by western blotting using FK2 antibody. **(B)** Ubiquitinated synaptotagmin was detected by western blotting in the enriched P3 and P2 synaptosomal fractions.

2. Summary of ubiquitinated proteins identified from rat brain

Over 1,500 proteins were identified in study. Selected proteins identified are listed below.

Cytoskeleton: actin, α/β-tubulins, neurofilaments medium and large, myosin6, GFAP, MAP1A/1B, MAP2, MAP6, α-adducin, septin4, spetin7, septin11, cadherin, β-catenin

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

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

Proteasomal subunits and UPS: Psmd1 26S non-ATPase subunit 1, Psmd1-p112 subunit, Psmc1-5 26S regulatory subunits Psmd2, Psmd3, E1, E2 and E3 ubiquitin ligases, UCHL1, UCHL5, USP24

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

Kinases: CaMKII-α/β, PKA, PKC, CaM kinase, MARK2, creatine kinase, phosphoglycerate kinase, Pfkm 6-phosphofructokinase, JNK2-β, CLK4, p35, STK38

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 I, amphiphysin 2, endophilin, Nsf Vesicle-fusing ATPase , Homer, SV2A, SV2B, spinophilin, clathrin heavy chain, AP2-α/β/μ, Shank1/3, AKAP5, PSD-95, NCAM, neurexin, 14-3-3 isoforms, NR2B, GluA1, GluA2, GluA4, Rabphilin3A, small GTPases, rabs, GAT-1, PICALM, Neurabin, Na(+)/K(+) ATPase alpha-1 subunit, , Munc-18, GNAO1

Extracellular matrix proteins: PNGase, neuroglycan C, DSD-1-proteoglycan, syndecan, chondroitin sulfate proteoglycan 5, Glypican-1

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

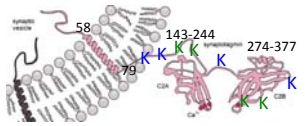
Nuclear proteins: Histones H1, H2A, H2B and H3, nucleolin like protein,

3. Summary of ubiquitinated peptides identified by mass spectrometry from rat brain tissue using KGG antibodies

PEP_SCORE	PEP_EXPECT	Modified lysine(s)	PEPTIDE_SEQUENCE	PROTEIN_ID	PROTEIN_NAME
Cytoskeletal proteins					
76.21	3.30E-07	K61	K.DSYVGDEAQSKR.G + GlyGly (K)	ACTB_RAT	Actin, cytoplasmic
24.73	0.1	K389	R.VRGESSEALKAEK.R + GlyGly (K)	MAP1A_RAT	MAP 1A
89.46	1.70E-08	K50	R.NLLSVAYKNVVGAR.R + GlyGly (K)	1433Z_RAT	14-3-3 protein zeta/delta
91.26	3.40E-08	K540	K.KNVTESPSFSAGDNPHVLYSPEFR.I + GlyGly (K)	MAG_RAT	Myelin-associated glycoprotein
84.16	1.40E-07	K60	K.TIGGGDDSFNTFFSETGAGKHVPR.A + GlyGly (K)	TBA1A_RAT	Tubulin alpha-1A
Kinases and Phosphatases					
24.04	0.18	K21	K.ELDQWIEQLNECKQLSESQVK.S + GlyGly (K)	PP2AA_RAT	PP2A catalytic subunit alpha
87.89	6.80E-08	K21	K.ELDQWVEQLNECKQLNENQVR.T + GlyGly (K)	PP2AB_RAT	PP2A catalytic subunit beta
61.44	0.0000046	K146	R.DLKPENLLLASKLK.G + GlyGly (K)	KCC2A_RAT	CAMKII subunit alpha
52.23	0.0003	K226	R.LYQQIKAGAYDFPSPEWDTVTPEAK.D + GlyGly (K)	KCC2A_RAT	CAMKII subunit alpha
84.53	1.10E-07	K31	K.DGDGTITTKELGTVMR.S + GlyGly (K)	CALM_RAT	Calmodulin
76.28	0.0000011	K22 + K31	K.EAFSLFDKDGDGTITTKELGTVMR.S + 2 GlyGly (K)	CALM_RAT	Calmodulin
33.4	0.021	K214	R.TWTLCGTPEYLAPEIILSKGYNK.A + GlyGly (K)	KAPCA_RAT	cAMP-dependent protein kinase catalytic subunit alpha
Synaptic proteins					
45.24	0.00087	K224	K.LILQTHQTKEGENIR.L + GlyGly (K)	IRK10_RAT	ATP-sensitive inward rectifier potassium channel
21.59	0.19	K23	K.TKQGVTEAAEK.T + GlyGly (K)	SYUB_RAT	Beta-synuclein
52.04	0.00014	K38	K.AEGAGTEEEGTQKESEPQAAADATEVK.E + GlyGly (K)	BASP1_RAT	Brain acid soluble protein 1
69.01	0.0000024	K235	K.IIDGGAAQKDGR.L + GlyGly (K)	DLG2_RAT	Disks large homolog 2
68.17	0.0000034	K258	R.SDPYHATTGPLSPSKQCGSPK.Y + GlyGly (K)	CXA1_RAT	Gap junction alpha-1
60.85	0.000012	K303	R.NYNKQASEQNWANYSAEQNR.M + GlyGly (K)	CXA1_RAT	Gap junction alpha-1
37.71	0.0041	K346	K.KVAAGHELQPLAIVDQRPSSR.A + GlyGly (K)	CXA1_RAT	Gap junction alpha-1
22.21	0.14	K136	K.FKYGIEEHGK.V + GlyGly (K)	CXA1_RAT	Gap junction alpha-1
94.16	9.60E-09	K13	K.VTFNSALAQKEAK.K + GlyGly (K)	ITM2B_RAT	Integral membrane protein 2B
43.31	0.0019	K30	K.GEAAAERPGEAAVASSPSKANGGENGHVK.V + 2 Deamidated (NQ); GlyGly (K)	MARCS_RAT	Myristoylated alanine-rich C-kinase substrate
23.31	0.19	K11	K.TAAKGEAAAERPGEAAVASSPSK.A + GlyGly (K)	MARCS_RAT	Myristoylated alanine-rich C-kinase substrate
40.12	0.002	K375	K.RPDEVPDAGPMKTNSTNPHK.D + 2 Deamidated (NQ); GlyGly (K)	NPTN_RAT	Neuroplastin
47.08	0.00021	K1700	K.APAAPKTPSK.A + GlyGly (K)	NRX2A_RAT	Neurexin-2-alpha
60.4	0.00001	K148	K.KLTPITVPQGLAMAKEIGAVK.Y + GlyGly (K)	RAC1_RAT	Ras-related C3 botulinum toxin substrate
54.29	0.00023	K595	R.LQVMIQPSEDIVKPENGPEQPQAGSSASKEAYI.- + GlyGly (K)	SC6A1_RAT	Sodium- and chloride-dependent GABA transporter
47.9	0.00026	L605	R.AAVPDAVGKCR.S + GlyGly (K)	AT1A1_RAT	Sodium/potassium-transporting ATPase subunit alpha-1
37.08	0.0036	K661	R.DAKACVVHGSDLK.D + GlyGly (K)	AT1A1_RAT	Sodium/potassium-transporting ATPase subunit alpha-1
29.51	0.014	K9	R.DKYEPAAVSEHGDK.K + GlyGly (K)	AT1A1_RAT	Sodium/potassium-transporting ATPase subunit alpha-1
45.67	0.00096	K40	R.MLQLVEESKDAGIR.T + GlyGly (K)	SNP25_RAT	Synaptosomal-associated protein 25
25.31	0.036	K96	K.LKSSDAYK.K + GlyGly (K)	SNP25_RAT	Synaptosomal-associated protein 25
63.95	0.000013	K70	K.HSAILASPNPDEKTK.E + GlyGly (K)	STX1A_RAT	Syntaxin-1A
42.16	0.0016	K104	K.NAINMKDVK.D + GlyGly (K)	SYT1_RAT	Synaptotagmin-1
27.36	0.029	K111	K.DLGKTMK.D + GlyGly (K)	SYT1_RAT	Synaptotagmin-1
21.05	0.14	K267	R.DLQSAEKEEQEK.L + GlyGly (K)	SYT2_RAT	Synaptotagmin 2
51.36	0.00018	K369	K.IGKNDAIGK.V + GlyGly (K)	SYT1_RAT	Synaptotagmin-1
103.23	2.20E-09	K47	R.DQKLSELDDRADALQAGASQFETSAAK.L + GlyGly (K)	VAMP3_RAT	Vesicle-associated membrane protein 3
74.43	0.0000018	K70	K.LSELDDRADALQAGASQFETSAAKLK.R + GlyGly (K)	VAMP3_RAT	Vesicle-associated membrane protein 3
41.82	0.0024	K70	R.ADALQAGASQFETSAAKLK.R + GlyGly (K)	VAMP3_RAT	Vesicle-associated membrane protein 3

SUMMARY

❖ Multiple sites of ubiquitination identified in synaptotagmin 1



❖ Ubiquitinated proteins were purified from rat brain tissue

❖ Over 1,500 ubiquitinated proteins were identified from brain tissue by mass spectrometry

❖ 430 GlyGly peptides in 172 unique protein identified using UBD's

❖ 1,718 GlyGly peptides in 286 unique proteins identified using KGG antibodies enrichment

❖ Also observed Asp and Glu residues surrounding site of ubiquitination as reported by Kim et al, 2011 using KGG antibody enrichment

❖ Ongoing studies examining activity-dependent changes in protein ubiquitination after exposure to drugs of abuse

REFERENCES

Hallet, P.J. et al (2008) Biochemical fractionation of brain tissue for studies of receptor distribution and trafficking. Current Protocols in Neuroscience. 1.16.1-16.

Lee, K.A., et al (2011) Ubiquitin ligase substrate identification through quantitative proteomics at both the protein and peptide levels. JBC. October 2011. Epub ahead of print

Kim, W., et al (2011) Systematic and Quantitative Assessment of the Ubiquitin-Modified Proteome. Mol. Cell. 44 (2) :p325-340

SUPPORT

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