SUPPLEMENTARY INFORMATION

Site-Specific Analysis of Protein *S*-Acylation by Resin-Assisted Capture (Acyl-RAC)

M.T. Forrester et al.

Address correspondence to: Dr. Patrick J. Casey, Box 2612, Duke University Medical Center, Durham, NC 27710. Tel: 919-684-6933; Fax: 919-684-6998; E-mail: casey006@mc.duke.edu

Supplementary Methods

Isolation of S-acylated peptides by Acyl-RAC and preparation for proteomic analysis – Resins containing immobilized protein(s) from the application of Acyl-Rac were suspended in 1 ml of 50 mM NH_4HCO_3 1 mM EDTA containing 0.5 μg Trypsin Gold® (Promega) and rotated at 37 °C at 37 °C for 12 h. Samples were then "tagged" with the 114 or 117 amu iTRAQ reagents (reconstituted in 70 μl EtOH) in 0.2 ml sodium borate pH 8.5 for 8 h. Following washing with 5 x 1 ml 100 mM HEPES, 1.0 mM EDTA, 1.0% SDS and 5 x 1 ml 10 mM NH_4HCO_3, equal amounts of resin samples were combined and washed three times with 650 μl 50 mM NH_4HCO_3. The resin was then resuspended in 200 μl of 50 mM NH_4HCO_3 containing 100 mM DTT and heated at 70° C for 30 min with shaking. Iodoacetamide was then added to a final concentration of 200 mM, and the suspension was incubated for 45 min at room temperature in the dark. The peptide mixture was processed using Proteospin® detergent removal kit (#23300, Norgen Biotek Corp.) per the manufacturer's instructions, except that peptides were released from the resin using 5% aqueous ammonia. Peptides were dried under reduced pressure and resuspended in 20 μl 0.1% v/v TFA with 2% v/v CH_3CN.

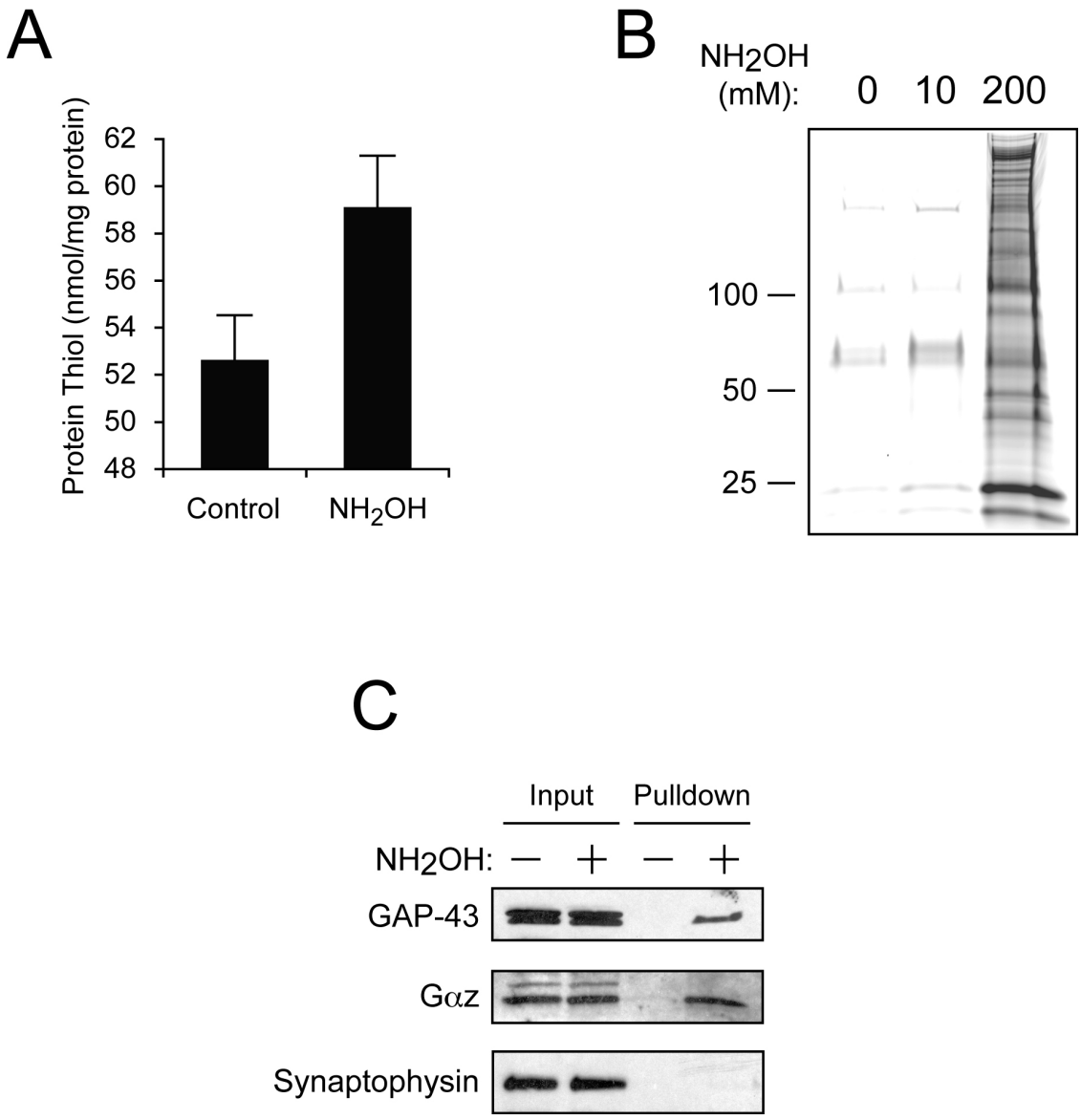
LC-MS/MS Analysis – Five μl of the sample was injected onto a 75 μm x 250 mm BEH C18 column (Waters) and separated using a 90 min gradient at a flow rate of 0.3 ml/min of 5 to

40% CH_3CN containing 0.1% formic acid. The outlet of the liquid chromatography column was coupled to an LTQ-Orbitrap hybrid mass spectrometer (Thermo). The Orbitrap MS/MS method utilized HCD fragmentation for peptide identification and quantification using iTRAQ reporter ions, based on Thermo application note 445 (http://www.thermo.com/eThermo/CMA/PDFs/Articles/articlesFile_9787.pdf). Briefly, the precursor scan method utilized profile mode and 60,000 resolution, with an automatic gain control (AGC) target of 1×10^6 and 2 microscans. MS/MS acquisition was performed on the top 3 precursor ions above a 5000 count threshold using high energy collision-induced dissociation (HCD) with a 3 Da isolation window, normalized collision energy of 45%, and 2 microscans. Product ion spectra were collected in profile mode with a resolution of 7500 and AGC target setting of 2×10^5. Dynamic exclusion settings were repeat count = 3, repeat duration = 30 sec, exclusion list = 250, and exclusion time = 120 sec.

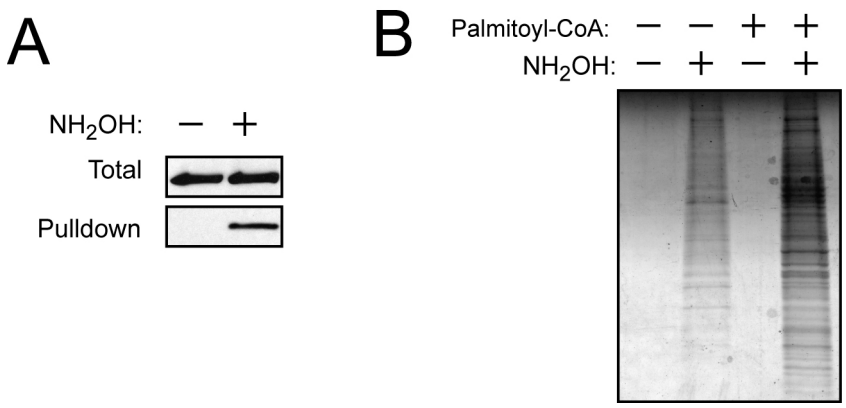
Peptide Identification and Quantitation – Mascot Distiller v2.3 (Matrix Science, Inc) was used was used to process raw Orbitrap LC-MSMS data, using standard parameters for high-resolution MS2 data except that deisotoping was turned off in the mass range from 112.5 to 121 m/z. Mascot generic (.mgf) files were searched on Mascot v2.2 (Matrix Sciences, Inc.), against Swissprot v57.10 database with human taxonomy (www.expasy.org). Search parameters were 10 ppm precursor and 0.02 Da product ion mass accuracy, with tryptic specificity and up to three missed cleavages. All samples were searched with iTRAQ4plex (K) and iTRAQ4plex (N-term) as fixed modifications and iTRAQ4plex (Y), Oxidation (M), and Carbamidomethyl (C) as variable modifications. Scaffold v2.6 Q+(Proteome Software Inc.) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 50.0% probability as specified by the Peptide Prophet algorithm.[1]

Peptide false-discovery rate (FDR) was determined to be 2.3% (2/88) by reverse database decoy search; after filtering for Cys-containing peptides, peptide FDR was 1.2% (1/84). Peptides were quantitated using the centroided reporter ion peak intensity, with the minimum peak height required for quantitation equal to 0.15% percent of the highest peak in the spectrum.

1. Keller, A.; Nesvizhskii, A. I.; Kolker, E.; Aebersold, R., Empirical statistical model to estimate the accuracy of peptide identifications made by MS/MS and database search. *Anal Chem* **2002,** 74, (20), 5383-92.



Supplementary Figure 1. Application of Acyl-RAC to detect *S*-acylation in purified bovine brain membranes. (*A*) Measurement of total thioester (S-acylation) by treating bovine membranes with either vehicle (control) or 200 mM NH_2OH, followed by thiol quantitation via the DTNB assay. Endogenous *S*-acylation was detected via Acyl-RAC, with analysis by (*B*) Coomassie staining or (*C*) immunoblotting for Gαz and GAP-43 (two known *S*-acylated proteins present in brain membranes), as well as Synaptophysin (not known to undergo *S*-acylation).



Supplementary Figure 2. Detection of endogenous *S*-acylation in cultured human cells. (*A*) Cultured T24 bladder carcinoma cells (2 x 10 cm dishes), processed in the absence (-) or presence (+) of 200 mM NH_2OH, were subjected to Acyl-RAC with immunoblotting for endogenous H-Ras. (*B*) A membrane-enriched lysate from HEK293 cells was incubated with either buffer or 100 μM palmitoyl-CoA for 1 h at 37 °C, and subjected to Acyl-RAC with analysis by Coomassie staining.

Supplementary Table 1. LC-MS/MS identifications of *S*-acylation by Acyl-RAC. Listed are triplicate analyses, along with mass error and Mascot scores. As listed in the supplementary methods, the false discovery rate was 2.3% based on a decoy database search.

Protein accession numbers	Peptide sequence	Cys?	Times ID x/3	Error (ppm)	Best Mascot Score
ACADL_HUMAN	AFVDNCLQLHEAK	YES	3	-0.12	50.1
ACTBL_HUMAN	CDVDIRK	YES	3	0.03	28.2
AKAP1_HUMAN	ADPAIKEPLPVEDVCPK	YES	1	1.74	42.1
ALDOA_HUMAN	ALANSLACQGK	YES	2	0.26	30.9
AT1A1_HUMAN	ACVVHGSDLK	YES	2	-0.72	44.1
AT1A1_HUMAN	KNCLVK	YES	3	-1.35	28.5
ATPG_HUMAN	GLCGAIHSSIAK	YES	3	-2.15	35.7
CCHL_HUMAN	GCPVNTEPSGPTCEKK	YES	3	-0.43	57.6
CD99_HUMAN	LCFKENAEQGEVDMESHR	YES	3	-1.47	51.7
CEPT1_HUMAN	CGDSHPESPVGFGHMSTTGCVLNK	YES	3	-1.10	60.2
CO4A1_HUMAN	AHGQDLGTAGSCLRK	YES	3	-0.34	34.5
CO4A1_HUMAN	HSQTIDDPQCPSGTK	YES	3	1.37	75
CO4A2_HUMAN	KFDVPCGGR	YES	3	0.99	59.8
CYC_HUMAN	CSQCHTVEK	YES	3	1.98	40.2
EFTU_HUMAN	GDECELLGHSK	YES	1	-1.07	27.4
EFTU_HUMAN	KGDECELLGHSK	YES	3	-1.46	43.2
ES1_HUMAN	NLSTFAVDGKDCK	YES	2	-1.05	34.2
FAS_HUMAN	CPPGVVPACHNSK	YES	3	-1.23	40.7
GNA11_HUMAN	TLESMMACCLSDEVKESK	YES	2	-1.89	52.8
GNAS2_HUMAN	GCLGNSK	YES	3	1.06	54
GNAS2_HUMAN	GCLGNSKTEDQR	YES	3	-1.58	36.8
GNAS2_HUMAN	GCLGNSKTEDQRNEEK	YES	3	-1.45	37.6
GRP75_HUMAN	RTIAPCQK	YES	3	-0.20	28.9
H2B1A_HUMAN	QVHPDTGISSK	NO	2	5.23	47.4
HNRL1_HUMAN	AIVICPTDEDLKDR	YES	2	-1.28	36.9
HNRL1_HUMAN	KAIVICPTDEDLKDR	YES	3	0.06	57.7
MBLC2_HUMAN	AGICHK	YES	2	3.82	30.6
MGST3_HUMAN	SGLGSGPKCCH	YES	3	0.57	47
MPP6_HUMAN	RDWDNSGPFCGTISSK	YES	2	-1.24	48.4
NFXL1_HUMAN	AGPECLHCEEGCSK	YES	3	0.02	43.6
NFXL1_HUMAN	CGHLCPAPCHDQALIK	YES	3	-1.20	48.6
NFXL1_HUMAN	FCPCQK	YES	2	-1.14	33.6
NFXL1_HUMAN	SCASPLWHCDQVCGK	YES	3	-1.21	54.9
NFXL1_HUMAN	TLPCGNHTCEQVCHVGACGECPR	YES	3	-1.23	55.7
ODPA_HUMAN	KGGCAK	YES	3	-2.74	33
PARK7_HUMAN	DVVICPDASLEDAKK	YES	2	-0.19	41.8
PDIA1_HUMAN	KEECPAVR	YES	3	-1.45	29.3

PGAM5_HUMAN	HLPGVCK	YES	3	-1.77	33.7
PSIP1_HUMAN	QPCPSESDIITEEDKSK	YES	1	6.87	33.2
PYR1_HUMAN	GLPVTCEVAPHHLFLSHDDLER	YES	3	-1.20	33.2
PYR1_HUMAN	SVHICHVAR	YES	3	1.02	44.9
RASH_HUMAN	KLNPPDESGPGCMSCK	YES	3	-2.53	36.8
RELL1_HUMAN	CTTEAEQDIEEEKVEK	YES	2	-2.13	33.1
RL10A_HUMAN	FSVCVLGDQQHCDEAK	YES	2	-1.35	50.3
RL15_HUMAN	HCGALR	YES	1	5.61	26.7
RL7A_HUMAN	TCTTVAFTQVNSEDKGALAK	YES	3	-0.88	32.7
RS11_HUMAN	KCPFTGNVSIR	YES	3	-1.37	61.2
RS16_HUMAN	TATAVAHCK	YES	1	2.85	28.7
RS20_HUMAN	KTPCGEGSK	YES	3	-2.21	56.3
RS23_HUMAN	ANPFGGASHAK	NO	2	5.04	25.1
RS5_HUMAN	KAQCPIVER	YES	3	-2.07	51.7
S38A2_HUMAN	SSVTHLLCASK	YES	2	-0.81	29
SC61B_HUMAN	KNASCGTR	YES	3	0.15	47.4
SND1_HUMAN	RGEFCIAK	YES	3	-0.93	26.5
SNP23_HUMAN	ETEKTLTELNKCCGLCVCPCNR	YES	3	-0.89	37.3
TIM50_HUMAN	VVVVDCKK	YES	3	-2.48	35.2
TSN14_HUMAN	EKCGVPFSCCVPDPAQK	YES	2	-2.00	34.3
UBA1_HUMAN	SIPICTLK	YES	1	0.95	31
ACADL_HUMAN	AFVDNCLQLHEAKR	YES	3	-0.79	18.3
AL3A2_HUMAN	HSFDTFSHQRPCLLK	YES	2	0.83	10.7
AT1A1_HUMAN	CSSILLHGK	YES	1	9.16	16.8
C1TM_HUMAN	GDAHECFVSPVAK	YES	1	0.50	21.1
CD151_HUMAN	VVPDSCCKTVVALCGQR	YES	1	-0.14	22
CD99_HUMAN	KLCFK	YES	1	1.10	14.7
CD99_HUMAN	KLCFKENAEQGEVDMESHR	YES	3	-1.34	29.3
EF1G_HUMAN	KAAAPAPEEEMDECEQALAAEPK	YES	1	4.97	15.2
ESTD_HUMAN	QISSNKCFGGLQK	YES	1	0.17	17.5
ESYT1_HUMAN	GSSVDAPPRPCHTTPDSQFGTEHVLR	YES	2	-0.68	17.1
G3P_HUMAN	AGAHLQGGAK	NO	3	4.34	24.5
IF5A1_HUMAN	GRPCK	YES	3	2.47	19.1
KI67_HUMAN	TTKIPCDSPQSDPVDTPTSTK	YES	1	-0.82	27.9
MYO1D_HUMAN	KLCASDK	YES	1	3.42	18
NFXL1_HUMAN	CNCGNTK	YES	1	0.50	16.2
NFXL1_HUMAN	EFKPPCGHK	YES	2	2.05	19.9
PREP_HUMAN	SQQSKPQDASCLPALK	YES	1	-1.54	18.6
RBP2_HUMA	KCELSK	YES	2	3.98	25
RL10_HUMAN	SCGKDGFHIR	YES	2	5.13	17.2
RL11_HUMAN	RTGCIGAK	YES	2	2.96	25.3
RL3L_HUMAN	KVACIGAWHPAR	YES	2	2.55	20.2
RS30_HUMAN	KVHGSLAR	NO	2	4.95	20.1
RT18A_HUMAN	KITGLCQEEHR	YES	1	2.54	20.5

SF01_HUMAN	CGGAGHIASDCK	YES	1	0.15	21
SNP23_HUMAN	TLTELNKCCGLCVCPCNR	YES	1	-1.74	15.2
SNP23_HUMAN	TTWGDGGENSPCNVVSK	YES	1	-0.57	17.9
TBL2_HUMAN	SGRPACQK	YES	2	3.47	22.5
TLCD1_HUMAN	SDFCPEHVPK	YES	2	-0.81	21.1
TM9S2_HUMAN	ETCKLVCTK	YES	2	0.52	21.6
ZN330_HUMAN	KCLSTHACACPLTDAECVECER	YES	1	0.94	16.2