LC/MS/MS analysis of SDS PAGE 1D gel bands – Identification of proteins following E-Selectin enrichment of a ZR-75-1 lysate.

Method

Lysates of ZR-75-1 cells were precipitated with E-selectin in the presence or absence of calcium and run on SDS page as described above and stained with silver nitrate. Eight bands of interest defined by comparing silver nitrate staining to Western blots with anti-sialyl Le^x of E-selectin IPs, were excised and subjected to in-gel reduction, alkylation and digestion with trypsin. Cysteine residues were reduced with dithiothreitol and derivatised by treatment with iodoacetamide to form stable carbamidomethyl derivatives. Trypsin digestion was carried out overnight at room temperature after initial incubation at 37^{o}C for 2 hours.

Peptides were extracted from the gel pieces by a series of acetonitrile and aqueous washes. The extract was pooled with the initial supernatant and lyophilised. Each sample was then resuspended in 23µL of 50mM ammonium bicarbonate and analysed by LC/MS/MS. Chromatographic separations were performed using an Ultimate LC system (Dionex, UK). Peptides were resolved by reversed phase chromatography on a 75 µm C18 PepMap column using a three step linear gradient of acetonitrile in 0.1% formic acid. The gradient was delivered to elute the peptides at a flow rate of 200 nL/min over 60 min. The eluate was ionised by electrospray ionisation using a Z-spray source fitted to a QTof-micro (Waters Corp.) operating under MassLynx v4.0. The instrument was run in automated data-dependent switching mode, selecting precursor ions based on their intensity for sequencing by collision-induced fragmentation. The MS/MS analyses were conducted using collision energy profiles that were chosen based on the mass-to-charge ratio (*m/z*) and the charge state of the peptide.

Database Searching: The mass spectral data was processed into peak lists using ProteinLynx Global Server v2.2.5 with the following parameters:

> MS survey – No background subtraction, SG smoothing 2 iterations 3 channels, peaks centroided (top 80%) no de-isotoping.

MS/MS – No background subtraction, SG smoothing 2 iterations 4 channels, peak centroiding (top 80%) no de-isotoping.

The peak lists was searched against the Swiss-Prot and a concatenated Swiss-Prot database using Mascot software v2.2 (http://www.matrixscience.com) using the following parameter specifications:

Precursor ion mass tolerance 1.2 Da

Fragment ion mass tolerance 0.6 Da

Tryptic digest with up to three missed cleavages

Variable modifications: Acetyl (Protein N-term), Carbamidomethylation (C), Gln->pyro-glu (N-term Q) and Oxidation (M).

Sequence information was obtained for all the peptides included in the results.

Results

The results of the analysis and database searches are given in Table 1 and the proteins precipitated with E-selectin in a calcium dependent manner in table 2.. Database generated files were uploaded into Scaffold 3 (v3_00_06) software (www.proteomesoftware.com) to create .sfd files (PR270 SJ gel Human Tax Only 21032011). All samples were aligned in this software for easier interpretation and used to validate MS/MS based peptide assignments and protein identifications. Peptide assignments were accepted if they contained at least two unique peptide assignments and were established at 100% identification probability by the Protein Prophet algorithm (Nesvizhskii, AI Anal. Chem. 2003 Sep 1;75(17):4646-58). The result table includes probability scores (Mowse) for each peptide identified from the protein sequence. The Threshold Identity Score corresponds to a 5% chance of incorrect assignment. Peptides identified below these probabilities were accepted following manual inspection of the raw data to ensure that fragment ions correctly match the assigned sequence.

Table 1: LC/MS/MS analysis of 1D SDS gel bands

Band No.	Protein I.D.	Accession No.	MW (Da)	Band No.	Protein I.D.	Accession No.	MW (Da)
SJ1_11+	Annexin A6	P08133	75826	SJ1_51-	Heat shock cognate 71 kDa protein	P11142	70854
	Heat shock cognate 71 kDa protein	P11142	70854		Heat shock protein 75 kDa, mitochondrial	Q12931	80060
	Heat shock protein 75 kDa, mitochondrial	Q12931	80060		Heat shock 70 kDa protein 1A/1B	P08107	70009
	Stress-70 protein, mitochondrial	P38646	73635		Actin, cytoplasmic 1	P60709	41710
	Heat shock 70 kDa protein 1A/1B	P08107	70009		Glyceraldehyde-3-phosphate dehydrogenase	P04406	36030
	Glyceraldehyde-3-phosphate dehydrogenase	P04406	36030		Tubulin beta-2C chain	P68371	49799
	Tubulin alpha-1B chain	P68363	50120		Aminopeptidase B	Q9H4A4	72549
	Ig gamma-1 chain C region	P01857	36083		Stress-70 protein, mitochondrial	P38646	73635
	Actin, cytoplasmic 1	P60709	41710		Transketolase	P29401	67835
	Annexin A2	P07355	38580		Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit 1	P04843	68527
	Trifunctional enzyme subunit alpha, mitochondrial	P40939	82947		Tubulin alpha-1B chain	P68363	50120
	Neuroblast differentiation-associated protein AHNAK	Q09666	628699		X-ray repair cross-complementing protein 6	P12956	69799
	E-selectin	P16581	66611		Prelamin-A/C	P02545	74095
	Tubulin beta-2C chain	P68371	49799		Trifunctional enzyme subunit alpha, mitochondrial	P40939	82947
	Prelamin-A/C	P02545	74095		Heat shock protein HSP 90-beta	P08238	83212
	78 kDa glucose-regulated protein	P11021	72288		V-type proton ATPase catalytic subunit A	P38606	68260
	6-phosphofructokinase, liver type	P17858	84964		Annexin A6	P08133	75826
	Aminopeptidase B	Q9H4A4	72549		Aspartyl-tRNA synthetase, mitochondrial	Q6PI48	73516
	Transketolase	P29401	67835		E-selectin	P16581	66611
	Heterogeneous nuclear ribonucleoprotein U	Q00839	90528		Ig gamma-1 chain C region	P01857	36083
	Bone marrow stromal antigen 2	Q10589	19756		Succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial	P31040	72645
	Aspartyl-tRNA synthetase, mitochondrial	Q6PI48	73516		L-lactate dehydrogenase A chain	P00338	36665
					Heterogeneous nuclear ribonucleoprotein R	O43390	70899

					Pyruvate kinase isozymes M1/M2	P14618	57900
					Heat shock protein HSP 90-alpha	P07900	84607
					Phosphoglycerate kinase 1	P00558	44586
					Lamin-B1	P20700	66368
					Long-chain-fatty-acid--CoA ligase 3	O95573	80368
					Delta-1-pyrroline-5-carboxylate synthase	P54886	87248
					Cofilin-1	P23528	18491
					Pyridoxal-dependent decarboxylase domain-containing protein 1	Q6P996	86652
					Elongation factor Tu, mitochondrial	P49411	49510
					Prolyl endopeptidase	P48147	80648
					Heterogeneous nuclear ribonucleoprotein U	Q00839	90528
					40S ribosomal protein	P46782	22862
					Xaa-Pro aminopeptidase 1	Q9NQW7	69873
					Glucose-6-phosphate isomerase	P06744	63107
SJ1_22+	Heat shock protein HSP 90-alpha	P07900	84607	SJ1_62-	Heat shock protein HSP 90-alpha	P07900	84607
	Heat shock protein HSP 90-beta	P08238	83212		Heat shock protein HSP 90-beta	P08238	83212
	Actin, cytoplasmic 1	P60709	41710		Actin, cytoplasmic 1	P60709	41710
	E-selectin	P16581	66611		Elongation factor 2	P13639	95277
	6-phosphofructokinase type C	Q01813	85542		Tubulin beta-2C chain	P68371	49799
	Tubulin alpha-1B chain	P68363	50120		Glyceraldehyde-3-phosphate dehydrogenase	P04406	36030
	Glyceraldehyde-3-phosphate dehydrogenase	P04406	36030		E-selectin	P16581	66611
	Tubulin beta-2C chain	P68371	49799		Tubulin alpha-1B chain	P68363	50120
	Galectin-3-binding protein	Q08380	65289		Transitional endoplasmic reticulum ATPase	P55072	89266
	Annexin A6	P08133	75826		Ig gamma-1 chain C region	P01857	36083
	Ig gamma-1 chain C region	P01857	36083		Aconitate hydratase, mitochondrial	Q99798	85372
	Elongation factor 2	P13639	95277		Heat shock protein 75 kDa, mitochondrial	Q12931	80060
	Delta-1-pyrroline-5-carboxylate synthase	P54886	87248		Pyridoxal-dependent decarboxylase domain-containing protein 1	Q6P996	86652
	Annexin A2	P07355	38580		Delta-1-pyrroline-5-carboxylate synthase	P54886	87248
	Aconitate hydratase, mitochondrial	Q99798	85372		Sodium/potassium-transporting ATPase subunit	P05023	112824

					alpha-1		
	Erlin-2	O94905	37815		Coatomer subunit beta'	P35606	102422
	Neuroblast differentiation-associated protein AHNAK	Q09666	628699		Elongation factor Tu, mitochondrial	P49411	49510
	Importin subunit beta-1	Q14974	97108		6-phosphofructokinase type C	Q01813	85542
	Elongation factor 1-alpha 1	P68104	50109		Importin subunit beta-1	Q14974	97108
	Protein transport protein Sec23B	Q15437	86424		Carnitine O-palmitoyltransferase 1, liver isoform	P50416	88311
	Glucose-6-phosphate isomerase	P06744	63107		Coatomer subunit alpha	P53621	138258
	DNA replication licensing factor MCM4	P33991	96498		Elongation factor 1-alpha 1	P68104	50109
	Cofilin-1	P23528	18491		Protein transport protein Sec23B	Q15437	86424
	Coronin-1B	Q9BR76	54200		Sorbitol dehydrogenase	Q00796	38300
	DNA replication licensing factor MCM7	P33993	81257		Eukaryotic initiation factor 4A-I	P60842	46125
	Pyridoxal-dependent decarboxylase domain-containing protein 1	Q6P996	86652		DNA replication licensing factor MCM4	P33991	96498
	Elongation factor Tu, mitochondrial	P49411	49510		Erlin-2	O94905	37815
	Endoplasmin	P14625	92411		L-lactate dehydrogenase A chain	P00338	36665
	Heterogeneous nuclear ribonucleoprotein U	Q00839	90528		Endoplasmin	P14625	92411
	Glutaminyl-tRNA synthetase	P47897	87743		Cofilin-1	P23528	18491
					DNA replication licensing factor MCM7	P33993	81257
					Heterogeneous nuclear ribonucleoprotein U	Q00839	90528
					Coronin-1B	Q9BR76	54200
					Phosphoglycerate kinase 1	P00558	44586
					Chloride intracellular channel protein 1	O00299	26906
					40S ribosomal protein S5	P46782	22862
					ATP synthase subunit alpha, mitochondrial	P25705	59714
					Glutaminyl-tRNA synthetase	P47897	87743
SJ1_33+	Tubulin beta-2C chain	P68371	49799	SJ1_73-	Tubulin beta-2C chain	P68371	49799
	Tubulin alpha-1B chain	P68363	50120		Actin, cytoplasmic 1	P60709	41710
	Glyceraldehyde-3-phosphate dehydrogenase	P04406	36030		Tubulin alpha-1B chain	P68363	50120
	E-selectin	P16581	66611		Puromycin-sensitive aminopeptidase	P55786	103211
	Actin, cytoplasmic 1	P60709	41710		Endoplasmin	P14625	92411
	Annexin A2	P07355	38580		Glyceraldehyde-3-phosphate dehydrogenase	P04406	36030
	Neuroblast differentiation-associated protein AHNAK	Q09666	628699		E-selectin	P16581	66611

	Ig gamma-1 chain C region	P01857	36083		Transitional endoplasmic reticulum ATPase	P55072	89266
	Alanyl-tRNA synthetase, cytoplasmic	P49588	106743		Heat shock protein HSP 90-beta	P08238	83212
	Ubiquitin carboxyl-terminal hydrolase 5	P45974	95725		Hexokinase-1	P19367	102420
	Neutral alpha-glucosidase AB	Q14697	106807		Ubiquitin carboxyl-terminal hydrolase 5	P45974	95725
	Puromycin-sensitive aminopeptidase	P55786	103211		Fatty acid synthase	P49327	273254
	Cofilin-1	P23528	18491		Pyruvate kinase isozymes M1/M	P14618	57900
	Bone marrow stromal antigen 2	Q10589	19756		Alpha-actinin-4	O43707	104788
	Heat shock protein HSP 90-alpha	P07900	84607		Ig gamma-1 chain C region	P01857	36083
					Alpha-actinin-1	P12814	102993
					Uncharacterized protein KIAA0090	Q8N766	111689
					Elongation factor Tu, mitochondrial	P49411	49510
					Ubiquitin-like modifier-activating enzyme 1	P22314	117774
					Catenin alpha-1	P35221	100009
					L-lactate dehydrogenase A chain	P00338	36665
					Alanyl-tRNA synthetase, cytoplasmic	P49588	106743
					Neutral alpha-glucosidase AB	Q14697	106807
					Cofilin-1	P23528	18491
					Staphylococcal nuclease domain-containing protein 1	Q7KZF4	101934
					Glucose-6-phosphate isomerase	P06744	63107
					Coronin-7	P57737	100542
					40S ribosomal protein S5	P46782	22862
					Chloride intracellular channel protein 1	O00299	26906
					Importin subunit beta-1	Q14974	97108
					DNA replication licensing factor MCM3	P25205	90924
					Exportin-T	O43592	109893
					Dihydrolipoyl dehydrogenase, mitochondrial	P09622	54143
					Extended synaptotagmin-1	Q9BSJ8	122780
SJ1_44+	E-selectin	P16581	66611	SJ1_84-	E-selectin	P16581	66611
	Ig gamma-1 chain C region	P01857	36083		Fatty acid synthase	P49327	273254
	Neuroblast differentiation-associated protein AHNAK	Q09666	628699		Tubulin beta-2C chain	P68371	49799
	Actin, cytoplasmic 1	P60709	41710		Actin, cytoplasmic 1	P60709	41710
	Tubulin alpha-1B chain	P6836	5012		Ig gamma-1 chain	P0185	3608

		3	0		C region	7	3
	Tubulin beta-2C chain	P68371	49799		Glyceraldehyde-3-phosphate dehydrogenase	P04406	36030
	Glyceraldehyde-3-phosphate dehydrogenase	P04406	36030		Heat shock protein HSP 90-beta	P08238	83212
	Fatty acid synthase	P49327	273254		Heat shock protein HSP 90-alpha	P07900	84607
	ATP-dependent RNA helicase A	Q08211	140869		ATP-dependent RNA helicase A	Q08211	140869
	Heat shock protein HSP 90-alpha	P07900	84607		Transitional endoplasmic reticulum ATPase	P55072	89266
	Coronin-1B	Q9BR76	54200		Pyruvate kinase isozymes M1/M2	P14618	57900
	Annexin A2	P07355	38580		Tubulin alpha-1B chain	P68363	50120

Table 2: List of proteins IP in a calcium specific manner.

Protein		ID	Size	Localisation	Function
Annexin A2	Human	P07355	38580	secreted	Membrane binding (Ca dependent)
Neuroblast differentiation-associated protein AHNAK	Human	Q09666	628699	nucleus	Neurona differentiation (Ca depedent)
78 kDa glucose-regulated protein	Human	P11021	72288	ER	Assembly of protein multimer
6-phosphofructokinase, liver type	Human	P17858	84964	Cytosol	Glycan catabolism
Bone marrow stromal antigen 2	Human	Q10589	19756	Cell membrane	Virus caption
Galectin-3-binding protein	Human	Q08380	65289	secreted	Cell adhesion