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Screening for EphB Signaling Effectors Using SILAC with a Linear Ion Trap-Orbitrap Mass Spectrometer

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Erythropoietin-producing hepatocellular carcinoma (Eph) receptors play important roles in development, neural plasticity, and cancer. We used an Orbitrap mass spectrometer and stable isotope labeling by amino acids in cell culture (SILAC) to identify and quantify 204 proteins with significantly changed abundance in antiphosphotyrosine immunoprecipitates after ephrinB1-Fc stimulation. More than half of all known effectors downstream of EphB receptors were identified in this study, as well as numerous novel candidates for EphB signaling.

Keywords: EphB • SILAC • mass spectrometry • phosphoproteomics • Orbitrap • IP

Introduction

Erythropoietin-producing hepatocellular carcinoma (Eph) receptors form the largest family of receptor tyrosine kinases (RTKs). There are 16 Eph genes in vertebrate genomes, and 14 of them are found in mammals.[1,2] Eph receptors are transmembrane proteins with conserved extra- and intracellular domains. The Eph family receptors are divided into two classes, EphA and EphB receptors, based on similarities in their extracellular domains and their binding preference for either glycosylphosphatidylinositol linked ephrinA ligands or transmembrane ephrinB ligands.

Activation of Eph receptors generally leads to cytoskeleton rearrangement in the cell. However, recent studies have shown that Eph signaling can have diverse cellular effects in addition to changes in cytoskeleton dynamics depending on the cellular contexts.[1] First identified as key regulators of axon guidance during development, Eph receptors have been found to play important roles in tissue patterning, angiogenesis, cell morphogenesis, neural plasticity and cancer.[1] Despite intensive studies over the last 20 years leading to more than 2000 publications in Pubmed, Eph signaling is still of a great research interest, and new effectors and mechanisms continue to be discovered frequently.

Quantitative proteomics by stable isotope labeling with amino acids in cell culture (SILAC) has quickly become a major tool for high throughput screening analysis for signaling pathways in recent years.[3] Particularly, in combination with antiphosphotyrosine (pY) immunoprecipitation (IP), SILAC has shown great promise for the investigation of signaling pathways downstream of RTKs.[4−6] In these studies, cells are first metabolically labeled with light or heavy amino acids during cell culture. After labeling, in one cell population, the RTK is activated to trigger tyrosine phosphorylation of downstream effectors. Then the lysates of the stimulated and the control cells are combined for anti-pY IP to pull down pY proteins together with their tight binding partners. The IPed proteins are then identified and quantified by MS and the relative abundance of the light and heavy versions of a protein is used to indicate whether the protein participates in the RTK pathway or not.

In a previous study, we used SILAC and quadrupole time-of-flight (QTOF) mass spectrometry (MS) to look for effectors in the EphB signaling pathway.[7] In that study, 46 out of 127 identified proteins were found to have changed abundance in pY IPs upon EphB receptor activation. Recently, the hybrid linear ion trap-Orbitrap (LTQ-Orbitrap) mass spectrometer has demonstrated great utility in proteomic research.[8−13] With high speed and sensitivity in tandem mass spectrometry (MS/MS) mode by the linear ion trap, this instrument has been used to identify many more proteins from complex peptide mixtures than a QTOF instrument using data-dependent switching to and from MS/MS.[14] Moreover, the high resolution of the Orbitrap can increase the accuracy of peptide quantitation. In this study, we have repeated the SILAC experiment using an LTQ-Orbitrap with the aim of identifying more candidate effectors in the EphB pathway.

Materials and Methods

Cell Culture and Cell Stimulation. Metabolic labeling and stimulation of cells were performed as previously described.[7] Briefly, 10 10-cm plates (approximately 10^8 total cells)/condition NG108 cells (mouse neuroblastoma × rat glioma hybrid) stably overexpressing EphB2 receptor[15] were differentially labeled in medium containing either normal or $^{13}C_6$ lysine and $^{13}C_6$ arginine (Cambridge Isotope Laboratories, Andover, MA). After five cell divisions to ensure nearly complete metabolic labeling, the cells were serum starved for 24 h. One cell population was treated with 2 μg/mL ephrinB1-Fc (Sigma-Aldrich) aggregated with anti-Fc IgG (Jackson Immunoresearch)

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for 45 min while the other population was treated with anti-Fc IgG aggregated Fc as a control. Cells were lysed in lysis buffer containing 1% Triton X-100, 150 mM NaCl, 20 mM Tris, pH8, 0.2 mM EDTA, 2 mM Na_3VO_4, 2 mM NaF, and protease inhibitors (Complete tablet; Roche, Mannheim, Germany).

Immunoprecipitation (IP) and SDS-PAGE. The "light" and "heavy" lysates were mixed in a 1:1 ratio (v:v) and incubated with agarose-conjugated anti-pY antibody PY99 (Santa Cruz Biotechnology, Santa Cruz, CA) for 4 h, and the beads were washed 4 times with lysis buffer. Precipitated proteins were eluted with a low pH buffer (pH2) containing 0.2% TFA/1% SDS. The eluates were neutralized with 1 M NH_4HCO_3 and separated by SDS-PAGE on a 7.5% Tris-HCl gel (Biorad). The gel was stained with Coomassie Blue and the gel lanes were cut horizontally into 10 sections for in-gel tryptic digestion.

In-gel Digestion. Gel bands were cut into small pieces and destained in 25 mM NH_4HCO_3/50% acetonitrile, dehydrated with acetonitrile and dried. Then the gel pieces were rehydrated with 12.5 ng/μL trypsin solution in 25 mM NH_4HCO_3 and incubated overnight at 37 °C. Peptides were extracted twice with 5% formic acid/50% acetonitrile followed by a final extraction with acetonitrile. Samples were concentrated by vacuum centrifugation to dryness and redissolved with 2% acetonitrile in 0.1% formic acid before further analysis.

Liquid Chromatography (LC)−MS/MS. An LTQ-Orbitrap hybrid mass spectrometer (Thermo Fisher Scientific) equipped with a nano-ESI source (Jamie Hill Instrument Services) was used for all LC−MS/MS analyses. A Nano-Acquity UPLC system (Waters) equipped with a 100-μm × 15-cm reverse phase column (Symmetry C18, Waters) was coupled directly to the ion trap instrument via a 10-μm-inner diameter PicoTip nanoelectrospray emitter (New Objective). Samples were loaded onto a trap column (180 μm × 2 cm Symmetry C18, Waters) with 2% acetonitrile in 0.1% formic acid for 4 min at 5 μL/min. After sample loading, the flow rate was reduced to 0.4 μL/min and directed through the analytical column, and peptides were eluted by a gradient of 6−40% acetonitrile in 0.1% formic acid over 120 min. Mass spectra were acquired in data-dependent mode with one 60 000 resolution MS survey scan by the Orbitrap and up to five concurrent MS/MS scans in the LTQ for the five most intense peaks selected from each survey scan. Automatic gain control was set to 500 000 for Orbitrap survey scans and 10 000 for LTQ MS/MS scans. Survey scans were acquired in profile mode and MS/MS scans were acquired in centroid mode. Mascot generic format files were generated from the raw data using DTASuperCharge (version 1.01) and Bioworks (version 3.2, Thermo Fisher Scientific) for database searching.

Protein Identification and Quantitation. Mascot software (version 2.1.0, Matrix Science, London, UK) was used for database searching. An IPI database containing mouse and rat protein sequences (downloaded January 01, 2007) was used. Peptide mass tolerance was 20 ppm, fragment mass tolerance was 0.6 Da, trypsin specificity was applied with a maximum of one missed cleavage, and variable modifications were $^{13}C_6$ Lys, $^{13}C_6$ Arg, oxidation of methionine, and phosphorylation of serine, threonine and tyrosine. To estimate the false positive rate for protein identification, a decoy database was created by reversing the protein sequences of the original database. On the basis of the decoy database search results, three filters for protein identification were applied: (1) Peptide score threshold was 20. (2) Protein score threshold was 40. (3) Each protein was identified based on at least two unique peptide sequences. The estimated false positive rate based on the decoy database search was 0.3%.

To merge the SILAC results of multiple gel fractions from the same sample preparation, all the identified peptide sequences from different gel fractions were combined and searched against the same IPI protein database to obtain the protein matches. Proteins identified based on the same set of peptides were grouped and reported as a single protein match. Proteins that were likely introduced during sample preparation were excluded from the reported protein list. These proteins included keratins and trypsin (from in-gel digestion), immunoglobins (from the PY99 antibody and Fc fusion protein), ephrinB1 (the stimulating ligand), ferritin, and serum albumin (from cell culture media).

SILAC quantitation was carried out using the open source software MSQuant (version 1.4.2a13) developed by Peter Mortensen and Matthias Mann at the University of Southern Denmark. The XIC intensities of the heavy and light peptides were measured, with the results verified by manual inspection of the MS spectra. The SILAC ratios of proteins were calculated by comparing the summed XIC intensities of all matched light peptides with those of the heavy peptides. As a loading control, a small volume of the combined lysates was subjected to in-gel digestion, LC−MS/MS analysis and the identified proteins were also quantified. The average ratio for all quantified proteins was used as a correction for ratios of proteins identified from the IP.

Phosphorylation Analysis. Phosphopeptides were identified using Mascot. All matched MS/MS spectra were inspected manually. In cases where there were multiple possibilities for the localization of the phosphates on the peptide, a simple statistical model[16] was used to calculate a score (Ascore) based on the number of matching ions for each possible localization using only the site determining ions.[17] We used an Ascore threshold of 19, which has been estimated to correspond to 99.5% confidence in site localization.[17] In addition, the results were filtered based on the intensity of the peak corresponding to the neutral loss of phosphoric acid (98 Da) from the precursor ion: The assignment was rejected if the peptide did not contain pS or pT and the intensity of the neutral loss peak was larger than 50% of the base peak.

Western Blotting. Proteins were separated by SDS-PAGE and transferred to PVDF membranes. Membranes were blocked with Tris buffered saline with Tween 20 containing 2% bovine serum albumin, incubated with the corresponding primary and horseradish peroxidase (HRP)-conjugated secondary antibodies (Santa Cruz Biotechnology), and visualized with ECL (Pierce Biotechnology, Rockford, IL). Anti-Hrs[18] and anti-STAM2[19] were kind gifts from Dr. Harald Stenmark. Anti-IRS2, anti-Erbin, PY99-HRP (Santa Cruz Biotechnology), anti-SASH1 (Abnova, Taiwan) and anti-EphB4 (R&D Systems, Minneapolis, MN) antibodies were used as indicated by the manufacturers.

Results and Discussion

Protein Identification and SILAC Quantitation. To screen for effectors in the EphB signaling pathway, we used a SILAC strategy that we employed in a previous study.[7] Briefly, two populations of NG108-EphB2 cells (NG108 cells that stably overexpress the EphB2 receptor) were differentially SILAC labeled with $^{13}C_6$ Lys/$^{13}C_6$ Arg or $^{12}C_6$ Lys/$^{12}C_6$ Arg. One cell population was treated with clustered ephrinB1-Fc to activate the EphB2 receptor while the other (control) population was treated with clustered Fc. After cell lysis, equal volumes of the

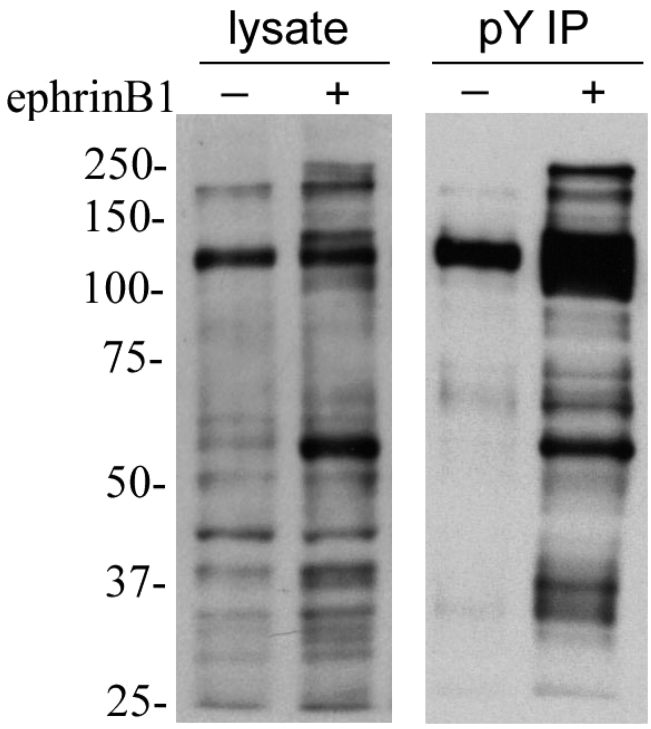

Figure 1. Tyrosine phosphorylation detected by anti-pY Western blotting of proteins in NG108 cell lysates and pY immunoprecipitates after ephrinB1 treatment. NG108-EphB2 cells were treated for 45 min with anti-Fc IgG aggregated ephrinB1-Fc or with anti-Fc IgG aggregated Fc as a control as indicated in the Materials and Methods. Total cell lysates and pY IPs were probed with PY99-HRP. Numbers to the left of the gel show MW $\times$ 10^{-3} based on protein MW standards.

two lysates were combined for anti-pY IP. Anti-pY Western blotting using the lysates and pY IPs indicated that several proteins were tyrosine phosphorylated after ligand stimulation (Figure 1). The IPed proteins were separated into 10 fractions using SDS-PAGE. Each fraction was digested with trypsin and analyzed by LC–MS with a hybrid LTQ-Orbitrap MS spectrometer for protein identification and quantitation. Coomassie staining of the gel before in-gel digestion revealed a faint haze of blue staining, but the gel contained insufficient protein for visualization of individual proteins (data not shown).

Two SILAC replicates (biological replicates) were carried out, in which 683 and 532 proteins were identified, respectively. Figure 2A shows the number of protein identifications in the two replicates.

Of the 804 proteins identified in the two SILAC replicates, 777 (672 from replicate 1 and 513 from replicate 2, 408 from both) were quantified and 27 were not able to be confidently quantified due to poor MS spectral quality (Figure 2B). The protein ratios from the two individual replicates were consistent (Figure 3). A list of the 777 quantified proteins is shown in Supporting Information Table 1.

Two-hundred and four proteins increased at least 1.5-fold in abundance in pY IP after ephrinB1 stimulation and 12 showed at least 1.5-fold decreased abundance. To further remove from the protein list redundancy caused by orthologous proteins from mouse and rat, proteins corresponding to the same gene name were clustered into a single entry. After clustering, 194 proteins showed at least 1.5-fold increased ratios and 10 showed decreased ratios. Table 1 lists the proteins with changed ratios.

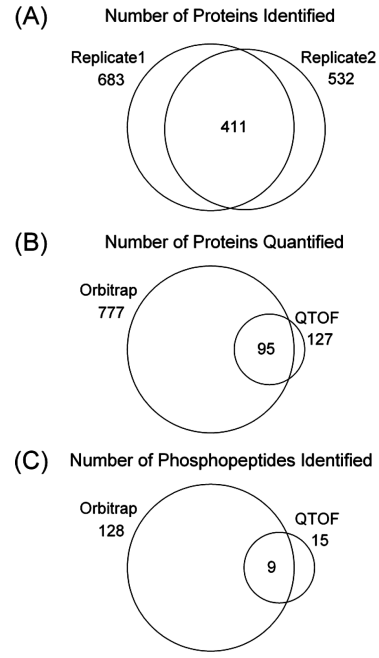

Figure 2. Identification of SILAC proteins and phosphopeptides from anti-pY IPs. (A) Venn diagram depicting the overlap in proteins identified in the two SILAC replicates by LTQ-Orbitrap. (B) Venn diagram depicting the overlap in proteins quantified in this study by Orbitrap and a previous study using a QTOF Micro mass spectrometer.[7] (C) Venn diagram depicting the overlap in phosphopeptides identified in this study by Orbitrap and the previous study using QTOF.

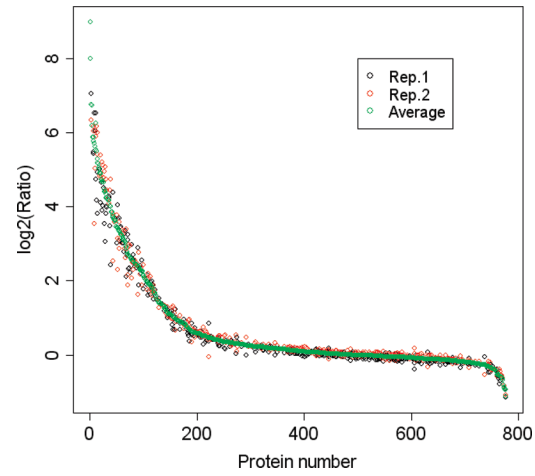

Figure 3. SILAC protein ratios from the two SILAC replicates. A total of 672 proteins from replicate 1 and 513 from replicate 2 were quantified. In total, 777 proteins were quantified, and 408 of them were quantified in both replicates.

Western Blotting Verification. To further verify the SILAC result from the MS analysis, Western blotting was carried out for five proteins with changed ratios whose antibodies were available. These proteins include: Hrs, STAM2, Erbin, IRS-2 and SASH1. None of these proteins have been previously reported to participate in Eph signaling except in our previous SILAC study[7] that found Hrs, STAM2, IRS-2 and SASH1. As shown in Figure 4, the Western blotting results for all the selected proteins were consistent with the corresponding SILAC ratios.

The NG108-EphB2 cell line has been used frequently in previous studies of ephrin/Eph signaling.[15,20–25] We have

Table 1. Proteins with at Least a 1.5 Change in Protein Abundance between Anti-Phosphotyrosine Immunoprecipitates from EphrinB1-Stimulated and Unstimulated NG108-EphB2 Cells[a]

no.	accession	protein name	mean ratio	Rep.1 CV	Rep.2 CV	CV (reps 1,2)	known EphB effector?
1	IPI00319843	Beclin-1	509				no
2	IPI00109667	nicotinamide nucleotide adenylyltransferase 1	255				no
3	IPI00222366	sterile alpha motif domain containing 5	107	76	1	37	no
4	IPI00556823	protein kinase, AMP-activated, alpha 1 catalytic subunit	106				no
5	IPI00752269	novel protein (possible orthologue of human Src homology 2 domain containing F (SHF))	76	54	3	23	no
6	IPI00330102	folliculin interacting protein 1	73	7			no
7	IPI00115056	trafficking protein particle complex 3	58	33			no
8	IPI00309259	partitioning-defective protein 3 homologue isoform 3	58	18	38	20	no
9	IPI00416163	unnamed protein product	55	21	38	16	yes
10	IPI00354665	apoptosis-stimulating protein of p53, 1	52	51	6	57	no
11	IPI00408219	N-chimaerin	49	33	19	24	no
12	IPI00749688	PREDICTED: similar to MGC114619 protein	46	15	36	28	no
13	IPI00331766	putative C3orf6 protein	45	10	19	38	no
14	IPI00127232	glutamate receptor interacting protein 1 isoform 1	39	8	31	36	yes[44]
15	IPI00338954	SAM and SH3 domain-containing protein 1	36	20	26	8	no
16	IPI00361275	PREDICTED: similar to TPR domain, ankyrin-repeat and coiled-coil-containing	35		16		no
17	IPI00213347	afadin	33	22	22	0	yes[24]
18	IPI00473693	Isoform 1 of Plakophilin-4	32	21			no
19	IPI00753111	PREDICTED: similar to Afadin (Af-6 protein)	30	22	18	3	yes[24]
20	IPI00359621	hypothetical protein LOC307833	30		13		no
21	IPI00761456	chimerin (chimaerin) 2	29	8	19	17	no
22	IPI00454039	Protein LAP2 (Erbb2-interacting protein) (Erbin)	27	12	16	2	no
23	IPI00125855	protein kinase C, delta	26	3			no
24	IPI00468418	signal transducing adaptor molecule2 (STAM2)	26	8	19	15	no
25	IPI00108870	Eph receptor B2	26	11	17	13	yes
26	IPI00565852	PREDICTED: similar to Eph receptor B3	25	12	15	3	yes
27	IPI00408892	RAB7, member RAS oncogene family	21	7	15	14	no
28	IPI00421832	dermcidin precursor	21	4	17	18	no
29	IPI00471127	Cdc42 effector protein 1 (Binder of Rho GTPases 5)	20	4	13	9	no
30	IPI00124742	eukaryotic translation initiation factor 4H	19	7	6	4	no
31	IPI00322033	target of myb1-like 2 isoform a	19	9	11	4	no
32	IPI00367930	PREDICTED: similar to Erbb2 interacting protein isoform 1	19		10		no
33	IPI00125534	Docking protein 1 (Downstream of tyrosine kinase 1) (p62(dok))	18	10	8	0	yes[15]
34	IPI00316623	catenin, delta 1 isoform 2	18	7			no
35	IPI00420753	PREDICTED: similar to SHB adaptor protein B	18	12	8	2	no
36	IPI00312067	inositol polyphosphate phosphatase-like 1, isoform CRA_c	18	9	6	1	yes[45]
37	IPI00315187	UPF0404 protein C11orf59 homologue	16	2	5	7	no
38	IPI00343984	coiled-coil domain containing 85B	16	8			no
39	IPI00187275	Carnitine deficiency-associated gene expressed in ventricle 3	16	2	5	15	no
40	IPI00202691	cancer susceptibility candidate 3	15		8		no
41	IPI00136475	Leucine-rich repeats and immunoglobulin-like domains protein 1 precursor (LIG-1)	14	7			no
42	IPI00331568	HGF-regulated tyrosine kinase substrate (Hrs)	14	6	6	1	no
43	IPI00153241	vacuolar protein sorting 37C	13		2	11	no
44	IPI00228877	connector enhancer of kinase suppressor of Ras 2	13	6			no
45	IPI00379844	Insulin receptor substrate 2 (IRS-2)	12	6	7	1	no
46	IPI00132135	midline 2	12	6			no
47	IPI00124298	Rho GTPase activating protein 5	12	6			no
48	IPI00366801	YTH domain family 2	12	4			no
49	IPI00134881	LIM domain-containing protein 1	11	4			no
50	IPI00323349	Tight junction protein ZO-2	11	4	4	4	no
51	IPI00137731	unnamed protein product	11	6	4	3	no
52	IPI00347255	Protein KIAA1688	11	7	2	8	no
53	IPI00336844	epsin 2	10	4			no
54	IPI00135971	tight junction protein 1	10	5	6	3	no
55	IPI00223987	leucyl/cystinyl aminopeptidase	10	4	4	4	no
56	IPI00364933	similar to signal transducing adaptor molecule 1 (STAM1)	9.9	5.0	3.7	1.4	no
57	IPI00130621	RAS p21 protein activator 1	9.6	1.4	3.4	1.9	yes[15]
58	IPI00660894	E3 ubiquitin-protein ligase CBL-B	9.5	3.8			no
59	IPI00229955	Ras association (RalGDS/AF-6) domain family 8	9.3	5.8			no
60	IPI00110435	nischarin	9.3	2.6	4.2	1.6	no
61	IPI00120433	SH2B adapter protein 2	8.9	2.7	2.0	1.1	no
62	IPI00480842	hypothetical protein LOC684097	8.6		2.4		no
63	IPI00221581	Eukaryotic translation initiation factor 4B (eIF-4B)	8.5	2.7	4.6	2.4	no
64	IPI00765594	noncatalytic region of tyrosine kinase adaptor protein 1 (predicted), isoform CRA_a	8.5	4.0	3.7	1.7	yes[15]
65	IPI00119809	lectin, galactoside-binding, soluble, 3 binding protein	8.3	3.9			no
66	IPI00363834	PREDICTED: similar to pleckstrin homology domain containing, family A member 6	8.2	3.0	2.8	2.9	no
67	IPI00121319	LIM only protein HLP	7.6	1.1	2.4	0.0	no
68	IPI00117375	syndecan binding protein isoform 1 (syntenin)	7.4	0.9	4.7	4.3	yes[43]
69	IPI00123505	Synaptophysin	6.9			0.6	no
70	IPI00116554	protein tyrosine phosphatase, nonreceptor type 11	6.6	3.8	1.6	4.1	yes[46]

Table 1. Continued

no.	accession	protein name	mean ratio	Rep.1 CV	Rep.2 CV	CV (reps 1,2)	known EphB effector?
71	IPI00154012	ubiquitin specific peptidase 15	6.5	3.0	1.4	2.9	no
72	IPI00128454	seizure related 6 homologue like 2	6.5	0.3	1.2	2.1	no
73	IPI00130185	protein phosphatase 1, catalytic subunit, alpha	6.4	1.4	2.5	1.8	no
74	IPI00133679	hypothetical protein LOC73711	6.2	0.5	0.9	0.0	no
75	IPI00117944	tumor susceptibility gene 101 protein	6.2	1.8	0.7	2.2	no
76	IPI00205566	calponin 3, acidic	6.0				no
77	IPI00229392	Ras-related GTP binding A	6.0	0.5	1.3	2.6	no
78	IPI00229434	tumor protein p53 binding protein, 2	5.9	3.5			no
79	IPI00323590	E3 ubiquitin-protein ligase CBL	5.8	1.4	1.3	0.2	yes[42]
80	IPI00136618	toll interacting protein	5.7	1.1		1.1	no
81	IPI00272559	Vav2 protein	5.6	1.1			yes[41]
82	IPI00381394	filamin C, gamma	5.5	1.9	2.4	0.5	no
83	IPI00133591	vacuolar protein sorting 28	5.5	0.8			no
84	IPI00308222	drebrin-like	5.5	1.0	2.0	0.1	no
85	IPI00227149	YTH domain family protein 3	5.4	1.7	1.3	0.2	no
86	IPI00272148	Cytohesin-3	5.4	1.0	1.2	0.4	no
87	IPI00132604	unnamed protein product	5.2	2.1	0.6	3.0	no
88	IPI00153207	unnamed protein product	5.2	0.1	2.8	2.2	no
89	IPI00325146	annexin A2	5.2		3.2		no
90	IPI00130883	Putative RNA-binding protein 3 (RNA-binding motif protein 3)	5.1		0.9		no
91	IPI00323483	programmed cell death 6 interacting protein	4.9	1.8	0.6	0.6	no
92	IPI00454019	unnamed protein product	4.9	1.5			no
93	IPI00458001	ataxin 2-like, isoform CRA_g	4.8	0.9	1.3	0.3	no
94	IPI00231715	protein phosphatase 1 gamma2	4.8		2.0		no
95	IPI00130115	Vesicle transport through interaction with t-SNAREs homologue 1B	4.8	2.4	0.6	1.6	no
96	IPI00626620	SEC24 related gene family, member C (S. cerevisiae), isoform CRA_b	4.8	1.3			no
97	IPI00230035	ATP-dependent RNA helicase DDX3X	4.4	0.7	1.9	1.4	no
98	IPI00553792	Isoform 2 of Caskin-1	4.4		2.0		no
99	IPI00114332	ribosomal protein S6 kinase polypeptide 1	4.3	1.2			no
100	IPI00132322	Trafficking protein particle complex subunit 5	4.1	1.0			no
101	IPI00402900	Isoform 1 of Engulfment and cell motility protein 2	4.1	0.5			no
102	IPI00222107	FERM domain containing 4A	4.0	0.5	0.4	0.3	no
103	IPI00264501	phosphatidylinositol-binding clathrin assembly protein	3.9	0.9			no
104	IPI00114613	Cdc42 binding protein kinase beta	3.9	1.2	0.3	0.8	no
105	IPI00331016	SEC24 related gene family, member B	3.8	0.6	1.0	1.1	no
106	IPI00420553	Serine/threonine-protein kinase TAO2	3.8				no
107	IPI00330862	Ezrin	3.7	1.0	1.1	0.7	no
108	IPI00221494	Lipoma-preferred partner homologue	3.7	0.1			no
109	IPI00313841	ATPase, H+ transporting, V0 subunit D isoform 1	3.7		0.6	0.9	no
110	IPI00118899	actinin alpha 4	3.7	0.6	1.0	0.3	no
111	IPI00380817	breakpoint cluster region homologue	3.6	0.0			no
112	IPI00223070	dedicator of cytokinesis 4	3.6	1.0	0.9	0.1	no
113	IPI00458995	polyubiquitin C	3.5	0.1	1.7	0.6	no
114	IPI00313275	Sorting nexin-9	3.5	0.7	0.4	0.3	no
115	IPI00132462	cytotoxic granule-associated RNA binding protein 1	3.3	0.0	0.8	0.6	no
116	IPI00206710	pleckstrin homology, Sec7 and coiled/coil domains 2	3.2		0.9		no
117	IPI00154057	protocadherin 1	3.2	0.7	0.8	0.2	no
118	IPI00380436	actinin, alpha 1	3.2	0.6	0.6	0.3	no
119	IPI00110247	TBC1 domain family member 15	3.0	0.5			no
120	IPI00464282	Hbs1-like (S. cerevisiae), isoform CRA_b	2.9	1.0			no
121	IPI00368041	similar to DNA-directed RNA polymerase II largest subunit	2.8		0.1		no
122	IPI00109334	Proto-oncogene tyrosine-protein kinase FER (p94-FER) (c-FER)	2.8	0.2		0.0	no
123	IPI00226727	Isoform 2 of Discs large homologue 2	2.8	0.6	0.9	0.0	no
124	IPI00189519	Histone H3.3	2.7				no
125	IPI00108150	Rho-associated protein kinase 2 (p164 ROCK-2)	2.7	0.6			no
126	IPI00117159	phosphatidylinositol 3-kinase, regulatory subunit, polypeptide	2.7	0.8	0.4	0.3	yes[39]
127	IPI00120923	Vacuolar protein sorting 16	2.6				no
128	IPI00380814	target of myb1 homologue	2.6	0.4			no
129	IPI00124753	misshapen-like kinase 1 isoform 2	2.6	0.5	0.8	0.0	no
130	IPI00380108	transmembrane protein 1	2.6	0.0			no
131	IPI00136498	lin 7 homologue c	2.6	0.3			no
132	IPI00558156	61 kDa protein	2.6	0.3	0.4	0.0	no
133	IPI00204923	ubiquitin specific peptidase 9, X chromosome	2.5		1.0		no
134	IPI00109932	DEAD (Asp-Glu-Ala-Asp) box polypeptide 6	2.5	0.4			no
135	IPI00123313	ubiquitin-activating enzyme E1, Chr X	2.5	0.6			no
136	IPI00123349	SEC23A	2.4	0.3	0.3	0.2	no
137	IPI00130423	Growth factor receptor bound protein 2-associated protein 2	2.4	0.3			no
138	IPI00462445	E3 ubiquitin-protein ligase NEDD4	2.3	0.9	0.1	0.2	no
139	IPI00133024	1110059P08Rik protein	2.2		0.6		no
140	IPI00114948	interferon induced transmembrane protein 2	2.2	0.3	0.4	0.3	no
141	IPI00457533	ubiquitin-associated protein 2	2.2	0.2	0.1	0.2	no
142	IPI00229895	dispatched homologue 2	2.2				no
143	IPI00331579	synaptogyrin 3	2.2		0.1		no
144	IPI00123292	Isoform 2 of Far upstream element-binding protein 1	2.2	0.3			no
145	IPI00226563	tweety homologue 3 (Drosophila), isoform CRA_a	2.1	0.5	0.3	0.2	no

Table 1. Continued

no.	accession	protein name	mean ratio	Rep.1 CV	Rep.2 CV	CV (reps 1,2)	known EphB effector?
146	IPI00329998	Histone cluster 1, H4h	2.1	0.2	0.2	0.0	no
147	IPI00116697	RAB6A, member RAS oncogene family	2.1	0.7	0.7	0.2	no
148	IPI00676192	latrophilin 2	2.1				no
149	IPI00111416	syntaxin 12	2.1	0.3	0.4	0.5	no
150	IPI00223253	heterogeneous nuclear ribonucleoprotein K	2.1	0.2	1.0	0.6	no
151	IPI00380824	MKIAA0144 protein	2.0	0.1	0.4	0.1	no
152	IPI00408378	Isoform 2 of 14−3−3 protein theta	2.0	0.3	0.4	0.0	no
153	IPI00762547	Isoform 1 of Intersectin-2	2.0	0.2	0.6	0.2	yes[31]
154	IPI00322492	Ewing sarcoma homologue	2.0	0.1	0.3	0.5	no
155	IPI00360064	PREDICTED: similar to Son of sevenless homologue 1	2.0			0.5	yes[20]
156	IPI00133428	protease (prosome, macropain) 26S subunit, ATPase 1	2.0				no
157	IPI00312527	Crmp1 protein	1.9	0.5	0.4	0.1	no
158	IPI00109375	Poliovirus receptor-related protein 2 precursor	1.9	0.2			no
159	IPI00125778	Transgelin-2	1.9		0.2		no
160	IPI00117039	Tyrosine-protein kinase ABL2	1.9	0.2	0.1	0.0	no
161	IPI00116112	Dynactin subunit 2	1.9	0.2			no
162	IPI00515195	eukaryotic translation initiation factor 4, gamma 1 isoform b	1.9		0.4		no
163	IPI00309413	noncatalytic region of tyrosine kinase adaptor protein 2	1.9	0.4	0.3	0.1	no
164	IPI00622847	Heterogeneous nuclear ribonucleoproteins A2/B1	1.9	0.2	0.2	0.4	no
165	IPI00221796	poly(rC) binding protein 2, isoform CRA_a	1.9		0.5		no
166	IPI00372054	PEF protein with a long N-terminal hydrophobic domain	1.9				no
167	IPI00135887	transmembrane protein 106B, isoform CRA_b	1.8	0.2			no
168	IPI00136917	Tyrosine-protein kinase-protein kinase SgK269	1.8		0.3		no
169	IPI00365284	eukaryotic translation initiation factor 4 gamma, 3	1.8				no
170	IPI00125298	SHC-transforming protein 1	1.8	0.3	0.2	0.0	yes[40]
171	IPI00420185	Epidermal growth factor receptor substrate 15-like 1	1.8	0.4	0.1	0.2	no
172	IPI00415402	Syntaxin-binding protein 1	1.8	0.4			no
173	IPI00463573	eukaryotic translation initiation factor 3 subunit 6 interacting protein	1.8	0.2			no
174	IPI00116966	Asparagine synthetase	1.8	0.3			no
175	IPI00226275	WD repeat domain 26	1.7	0.1			no
176	IPI00406118	NS1-associated protein 1 isoform 2	1.7	0.3	0.0	0.1	no
177	IPI00553633	cullin 7	1.7	0.3			no
178	IPI00129417	heterogeneous nuclear ribonucleoprotein D-like	1.7	0.1		0.2	no
179	IPI00227392	14−3−3 protein eta	1.7	0.2	0.5	0.3	no
180	IPI00470095	G protein-coupled receptor kinase-interactor 1	1.7	0.3	0.1	0.1	no
181	IPI00114401	emerin	1.6		0.3	0.5	no
182	IPI00128202	eukaryotic translation initiation factor 3, subunit 3 (gamma)	1.6		0.1		no
183	IPI00308162	solute carrier family 25 (mitochondrial carrier, Aralar), member 12	1.6	0.4			no
184	IPI00280250	SH3 and PX domains 2A	1.6				no
185	IPI00321647	eukaryotic translation initiation factor 3, subunit 8	1.6	0.1	0.1	0.1	no
186	IPI00118384	14−3−3 protein epsilon (14−3−3E)	1.5	0.1	0.2	0.1	no
187	IPI00230704	betaPix-c	1.5	0.1			no
188	IPI00229548	Solute carrier family 1 (neutral amino acid transporter), member 5	1.5	0.1			no
189	IPI00331334	Bcl-2-binding protein Bis	1.5	0.0	0.0	0.1	no
190	IPI00230707	14−3−3 protein gamma	1.5	0.2	0.1	0.0	no
191	IPI00114560	Ras-related protein Rab-1A	1.5	0.0	0.1	0.2	no
192	IPI00131329	Sorting nexin-18	1.5	0.1			no
193	IPI00116498	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide	1.5	0.1	0.1	0.0	no
194	IPI00362014	Tln1 protein	1.5				no
195	IPI00230445	peripherin	0.68	0.04	0.09	0.13	no
196	IPI00331738	52 kDa Ro protein	0.67	0.03	0.07	0.08	no
197	IPI00222801	Neuronal proto-oncogene tyrosine-protein kinase Src	0.67	0.12			no
198	IPI00117821	Breast cancer antiestrogen resistance protein 1 (p130cas)	0.66	0.05	0.07	0.02	yes[47]
199	IPI00113563	Focal adhesion kinase 1 (FADK 1) (pp125FAK)	0.64	0.06	0.05	0.02	yes[47,48]
200	IPI00135965	Alpha-internexin	0.57	0.04			no
201	IPI00137970	SH2 domain containing 3C	0.55	0.05	0.04	0.01	yes[49]
202	IPI00223751	Rho GTPase activating protein 12 isoform 1	0.52	0.05	0.05	0.01	no
203	IPI00330231	Rap guanine nucleotide exchange factor (GEF) 1 isoform 3	0.52	0.05			no
204	IPI00111258	unnamed protein product	0.46	0.04	0.04	0.01	no

[a] For proteins identified based on the same set of peptides, only one protein is shown in this table for each protein group. More detailed information about each protein group is included in Supporting Information Table 1.

observed from our previous SILAC study[7] that the NG108-EphB2 cells express endogenous EphB4 and EphB3 receptors and upon ephrinB1 stimulation these receptors are activated along with EphB2. This observation was confirmed in this study. To verify that EphB receptors other than EphB2 are expressed in NG108 cells, wild type NG108 cells were treated with ephrinB1 using the same procedure as the SILAC experiment. Lysates and pY IPs were probed with anti-EphB4 and anti-pY antibodies. The anti-EphB4 blot (Figure 4) indicates that EphB4 is expressed in wild type NG108 cells and can be tyrosine phosphorylated upon ligand treatment. The anti-pY blot on the lysates and pY IPs did not show a detectable difference between the control and stimulated cells due to interference from basal signals (data not shown), suggesting

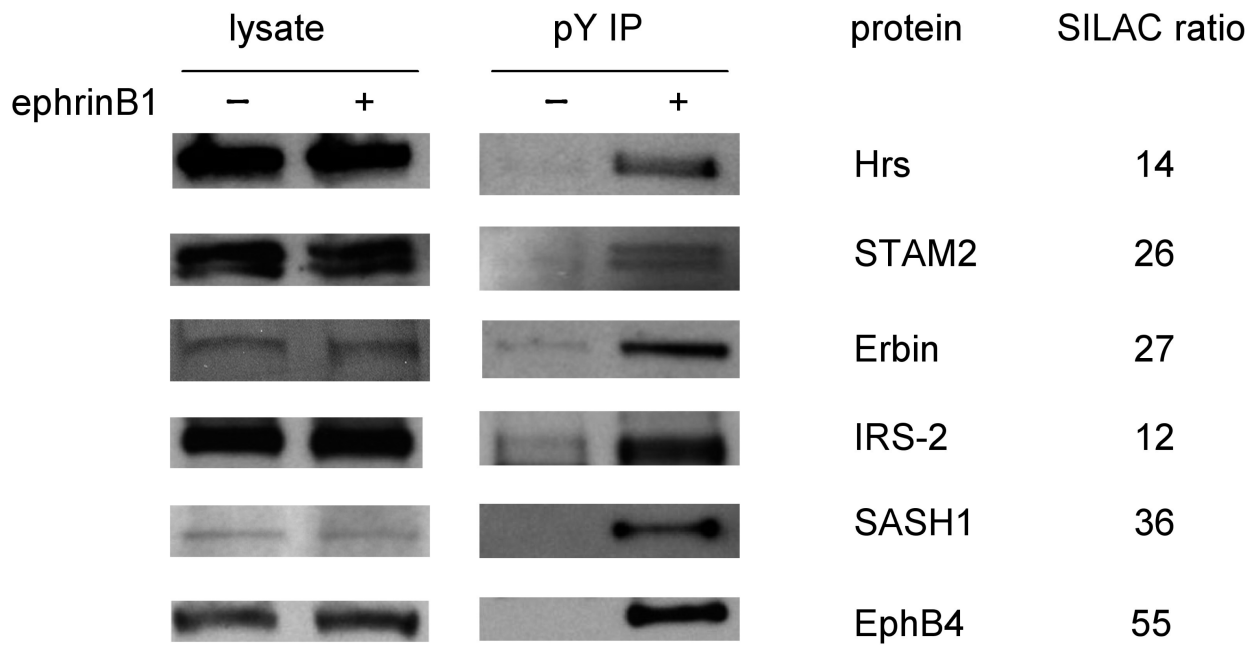

Figure 4. Western blotting analysis of selected candidate effector proteins. Cells were cultured and stimulated with ephrinB1 in the same way as in the SILAC experiments. The whole cell lysates and anti-pY IPs were probed with the indicated antibodies. For the EphB4 receptor, wild type NG108 cells were used. For all other proteins, NG108-EphB2 cells were used.

the effect of Eph signaling in wile type NG108 cells is much subtler than in the NG108-EphB2 cells. On the basis of these new findings, care should be taken when using NG108 cell lines for Eph signaling studies as well as interpreting results from previous studies in which these cell lines were used.

Phosphorylation Sites. The SILAC experiment identified 128 unique phosphopeptide sequences using Mascot. Because most of these phosphopeptides contain multiple serine/threonine/tyrosine residues, we tried to localize the phosphorylation sites using a simple statistical model for matching site-determining ions in the MS/MS spectra.[16,17]

Using this model, we localized 116 phosphorylation sites (38 pS, 12 pT and 66 pY) on 115 peptides. To determine how many of the identified phosphorylation sites are novel, the Swiss-Prot knowledgebase (downloaded September 03, 2007) and data sets of phosphorylation sites from several major large scale proteomics studies[26−30] were searched using in-house written Perl scripts. Sixty-seven of the 116 localized phosphorylation sites were not found in these databases (Supporting Information Table 2). The annotated MS/MS spectra of all identified phosphopeptides are included in Supporting Information File1 and File2. File1 contains spectra of phosphopeptides whose phosphorylation sites were localized by Ascores. File2 contains spectra of phosphopeptides whose phosphorylation sites could not be localized by Ascores. For these peptides, the phosphorylation sites corresponding to the best Ascores were used to annotate the fragment ions in the spectra. All the identified phosphopeptides together with their Ascores are listed in Supporting Information Table 2. It is possible that the phosphotyrosine sites on proteins with ratio changes are regulated by the EphB activity, but due to the fact that almost all phosphoproteins have multiple phosphorylation sites, a protein ratio change from anti-pY IP cannot be attributed to the change of a specific pY site. A quantitative experiment that does not involve pY protein IP (for example, phosphopeptide enrichment after digestion of the whole cell lysate) would be needed to confirm the link between specific pY sites and EphB signaling.

Comparison between the LTQ-Orbitrap Results and Previous QTOF Results. Previously we have carried out a similar SILAC study using an older QTOF instrument (Micromass, QTOF-Micro, installed in 2003).[7] Figure 2B and C shows a comparison of the numbers of protein identifications and phosphopeptide identifications in the two studies. Using approximately the same number of cells, the LTQ-Orbitrap analysis identified 5 times more proteins and 10 times more phosphopeptides than the QTOF Micro. This is largely attributed to the high sensitivity and sequencing speed afforded by the LTQ-Orbitrap as has been documented by previous studies.[10,14] When considering these results, it should be kept in mind that the Orbitrap SILAC experiment was performed twice, whereas the QTOF experiment was performed once, which would slightly exaggerate the difference in number of proteins quantified. Ninety-five of the 127 proteins that were identified in the QTOF experiment were identified and quantified in the Orbitrap study. Supporting Information Figure 1 shows the protein ratios measured by the QTOF and the Orbitrap. While the majority of these proteins have consistent ratios in the two studies, we did observe that 13 of the 95 proteins changed by more than 1.5-fold in one experiment but not the other. These proteins are listed in Supporting Information Table 3. However, for most of these proteins, the two ratios are close to the cutoff ratio of 1.5. A summary comparison of the proteins identified in both Orbitrap experiments as well as the previous QTOF study is shown in Supporting Information Table 4.

Differences in SILAC ratios between the two studies can be attributed to two major reasons. First, although the same protocol for cell culture and treatment was used, the two experiments were performed more than two years apart and the intensities of Eph activation may have been slightly different in the two studies (biological variation). For example, the

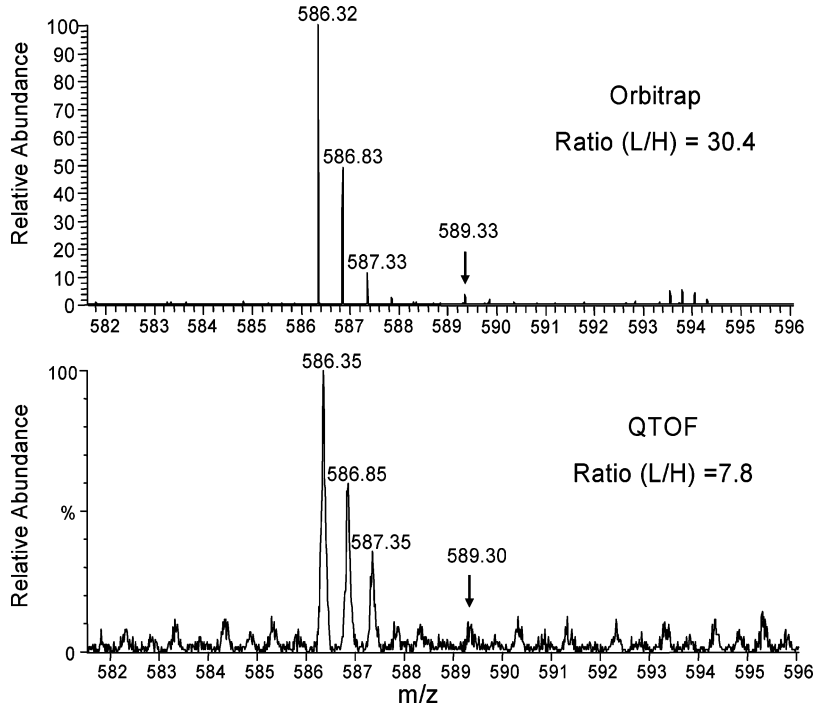

Figure 5. Measurement of SILAC peptide ratios using LTQ-Orbitrap and QTOF Micro mass spectrometers. The identified peptide (LLVDNQGLSGR) was from SAM and SH3 domain-containing protein 1 (SASH1, IPI00338954). The peptide ratio was calculated as the sum of intensities of the first three isotopic peaks of the light peptide over the sum of the heavy peptide peak intensities. The arrow in each panel indicates the monoisotopic peak for the heavy peptide.

protein Intersectin, which is a known effector downstream of EphB,[31] has a SILAC ratio of 0.99 in the QTOF study and 2.0 in the Orbitrap study. Second, the QTOF Micro and Orbitrap instruments have different dynamic ranges for quantitation. It was observed that the SILAC ratios measured by the Orbitrap were generally more dramatic than the ratios from the QTOF (Supporting Information Figure 1). We attribute this to the high dynamic range of Orbitrap.[32] Due to the different detection principles employed, the two instruments have quite different characteristics of spectral noise. As shown in Figure 5, the Orbitrap spectrum has lower background signal than the QTOF spectrum. When the peak intensity is low the background can contribute considerably to the ratio obtained by automated SILAC quantitation.

Comprehensiveness of the Screen. An extensive literature search using Pubmed found 42 key signaling proteins that are known to be close EphB binding partners or regulated by activation of EphB receptors (Supporting Information Table 5). Twenty-four of these were identified in this study, and 17 of the 24 proteins had SILAC pY IP abundance changes of 1.5 or more. Seven proteins had pY IP abundance changes upon ephrin stimulation of less than 1.5-fold, but for most of them the directions of the subtle ratio changes were consistent with previous studies, such as PI3K, Grb2 and MAPK. The fact that more than half of all known effectors in EphB signaling were identified in this single study suggested the power of the strategy and the comprehensiveness of the screen. These known effectors only account for a small proportion (~10%) of the proteins with changed ratios identified in this study, suggesting many more novel candidates may participate in EphB signaling with their roles yet to be characterized.

The reasons why many known effectors were not identified in this screen may include: (1) Eph signaling has versatile functions. Many known effectors and signaling effects are only observed in specific cell types and cellular contexts, for example, the NMDA receptor in neurons[33] and ZAP70 in T cells.[34] (2) The known effectors are based on studies on any of the 6 EphB receptors while only three of them (EphB2, EphB3 and EphB4) are known to be expressed in the cell line that was used in this study. (3) It has been shown that different intensities of Eph signaling can produce very different cellular effects.[1] Therefore regulation of effectors is dependent on intensity of Eph receptor activation, which is in turn dependent on level of receptor expression and concentration/affinity of stimulating ligands etc. (4) Different effectors may have different time courses of activation and the activation of specific effectors may only be observed at a specific time point. (5) Some effectors may not contain tyrosine phosphorylation sites or have low binding affinity/stoichiometry to other effectors. (6) Some effectors, for example, Src family kinases including Src, Fyn and Yes, are tyrosine phosphorylated at different sites both when activated and inhibited.[35] In this case the overall protein phosphorylation level, i.e. the SILAC ratio, might not reflect the level of activation. (7) The amounts of the effector proteins, in the context of the other proteins being analyzed, were below the detection limit of the mass spectrometer.

Gene Ontology (GO) Analysis. We performed a GO analysis of the quantified proteins using a commercial tool from ProteinCenter (Proxeon) for annotating and comparing protein data sets. The quantified proteins were first classified into two groups: proteins with ratio changes and proteins without ratio changes. GO analysis was performed for both groups using biological processes, cellular components and molecular functions classifications (Figure 6).

In theory, all proteins with changed ratios were pulled down in pY IPs due to specific binding and are specific to EphB signaling. Pulldown of proteins with no ratio changes could be due to either specific (some tyrosine phosphorylated

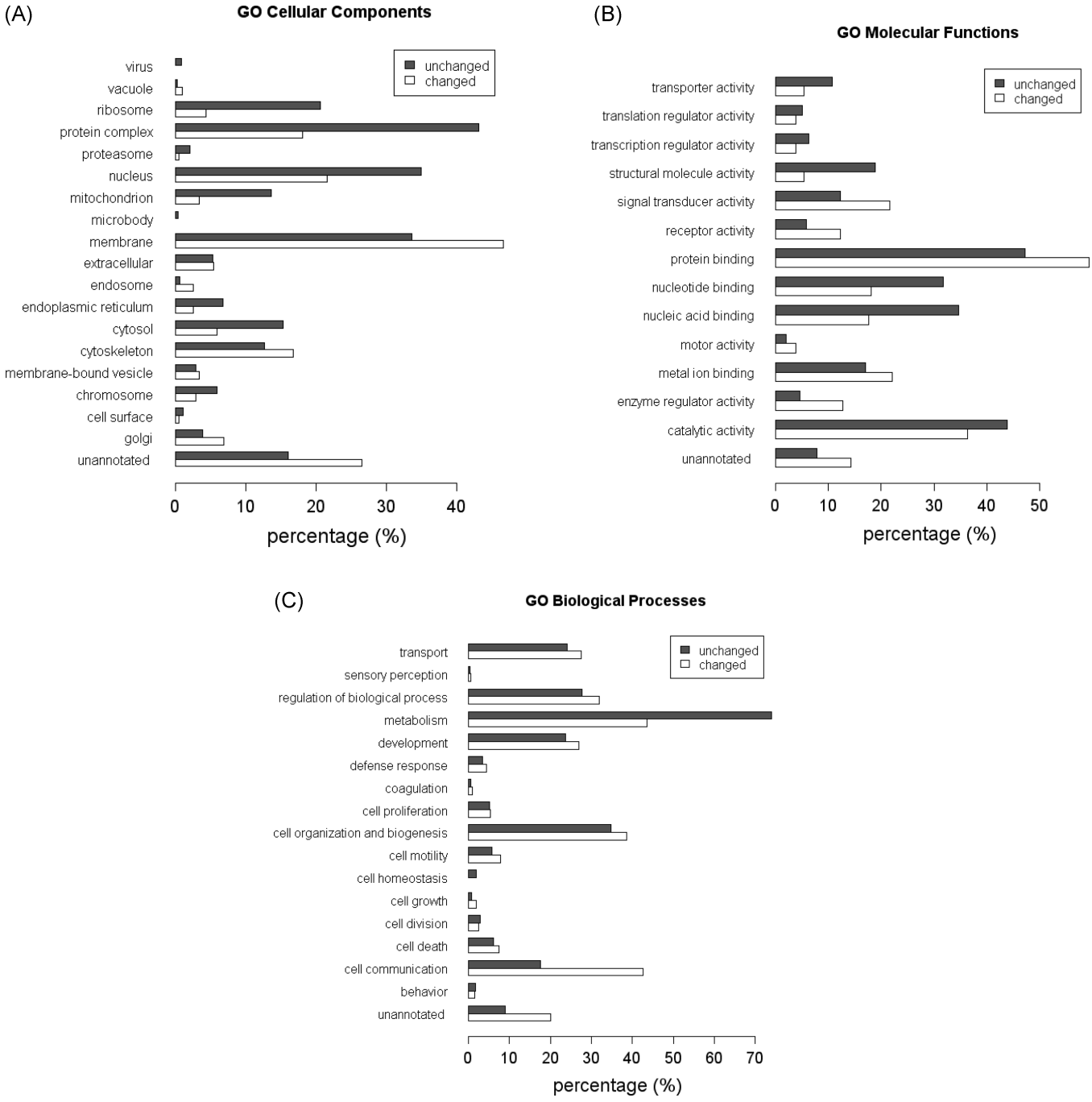

Figure 6. GO analysis of proteins with changed and unchanged SILAC ratios. All quantified proteins were classified into two groups: proteins with ratio changes less than 1.5 fold (unchanged) and proteins without ratio changes greater 1.5 (changed). GO analysis was performed for both groups on (A) cellular components, (B) molecular functions, and (C) biological processes.

proteins do not change their phosphorylation status after Eph activation) or nonspecific binding during IP. Therefore enrichment of a specific GO annotation in one group can indicate a general difference between the Eph signaling specific/nonspecific proteins or pY IP specific/nonspecific proteins.

The GO cellular components analysis (Figure 6A) shows that proteins from ribosome, nucleus, mitochondrion, and endoplasmic reticulum (ER) are enriched in the unchanged protein group, suggesting that a considerable number of nonspecific binding proteins from the pY IP came from these organelles. GO molecular functions analysis (Figure 6B) shows more nucleic acid/nucleotide binding proteins in the unchanged protein group, which also suggests the nucleus and ribosome as major sources of nonspecific binding proteins. This implies that more vigorous clarification of lysate before pY IP, e.g. centrifugation at higher speed or for longer time, which can better remove these organelles, may reduce IP background and promote identification of more specific target proteins. Another observation is that membrane proteins are enriched in the changed groups, suggesting significant Eph signaling occurs on the cellular membrane.

From the GO molecular functions/biological processes analysis (Figure 6B and C), proteins in the categories of cell communication and signal transduction are enriched in the changed proteins, while proteins in the categories of metabolism and structural activity are enriched in the nonchanged

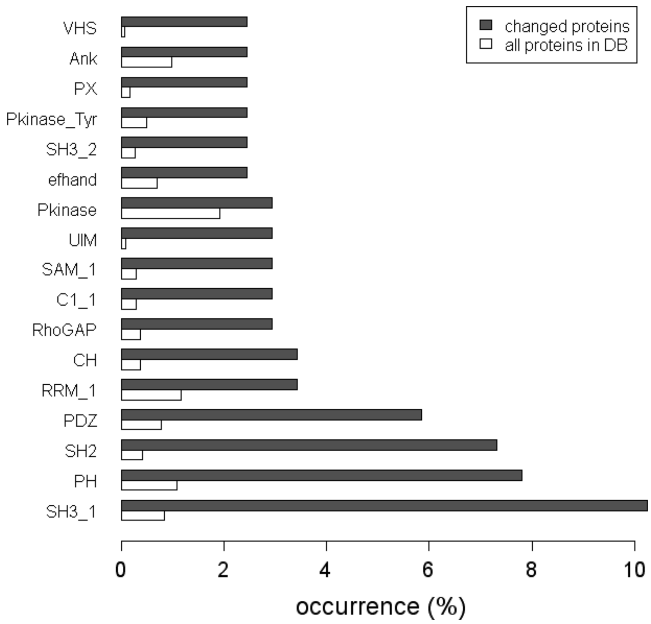

Figure 7. Domain analysis for proteins with SILAC ratio changes. Domain information was obtained from the Pfam annotation in the IPI cross-reference files. Domains that occur in at least 5 (out of 204) proteins are included in the figure. The occurrence of all domains in the combined IPI mouse and rat database is used as a control.

proteins. This is consistent with the established notion that the SILAC strategy we employed is an effective way to identify specific signaling target proteins from a background of nonspecific interactions, which might be expected to be dominated by highly abundant housekeeping/structural proteins.

In all three GO analyses, the unannotated proteins are more enriched in the regulated proteins category, suggesting that many of the proteins with changed SILAC ratios have not been well studied.

Domain Analysis. The presence of conserved structural domains in a protein can suggest particular functions for the protein. Therefore domain analysis can be used as a preliminary search for potential protein groups with specific functions or interactions in a signaling pathway.

The cross-reference files for the IPI mouse and rat protein databases were searched to obtain the Pfam domain annotations for the all quantified proteins (Supporting Information Table 1). For the proteins with changed SILAC ratios upon ephrinB1 stimulation (shown in Figure 7), it is clear that several specific domains are overrepresented: (1) SH3 (including SH3_1 and SH3_2), PH, CH and RhoGAP domains are known to be indicative of a protein involved in signal transduction related to cytoskeletal organization, which is consistent with the consensus that cytoskeleton rearrangement is one major outcome of EphB signaling. (2) SH2 domains, which are important regulatory modules of tyrosine phosphorylation-dependent signaling cascades, are also overrepresented. (3) The EphB receptor has a PDZ binding motif at the C-terminus and is known to bind to PDZ-domain containing proteins including Syntenin, Afadin, and Grip1. All these proteins were identified in this SILAC study as proteins with changed ratios. In addition, nine proteins with changed SILAC ratios were found to contain PDZ domains, which are possible novel binding partners of EphB receptors through their PDZ domains. (4) UIM and VHS domains are also overrepresented, suggesting many effectors may be involved in vesicular trafficking/protein degradation triggered by ubiquitination of Eph receptors.

Biological Implications of the Novel Candidate Effectors. Of the proteins found to be involved in EphB signaling in this study but not our previous study, we validated Erbin by Western blotting (Figure 4). Lending further support to the validity of our results, nine of the proteins found in this but not our previous study have been reported by others to be involved in ephrin signaling. These proteins include Intersectin,[31] PI3K,[36−39] Shc,[40] Vav2,[41] Cbl,[42] Syntenin,[43] Grip1,[44] Grb2,[40] and Sos1.[20] In addition, in-silico protein function and interaction analysis was performed by feeding the list of proteins of changed SILAC ratios into Ingenuity Pathways Analysis software (Ingenuity Systems, http://www.ingenuity.com), which searches existing literature and interaction databases (including Ingenuity curated findings, BIND, BIOGRID, DIP, INTACT, Interactome studies, MINT and MIPS, all downloaded 07/08/2008) for protein interaction and regulation networks. 98 of the 204 proteins with changed SILAC ratios could be assigned to a single interaction network based on previously reported direct protein−protein binding (Supporting Information Figure 2), thus supporting the ability of our screen to find functionally related proteins. The software was also able to classify the proteins into eleven major signaling networks (each network included a minimum of 10 proteins from our list of proteins that change in response to ephrinB addition) with relatively independent functions (Supporting Information Figure 3). These networks are mainly involved in regulation of cell morphology, cellular assembly and organization as well as development, which are consistent with known functions of EphB signaling. The same analysis was also carried out for the 46 proteins with changed SILAC ratios found in the QTOF analysis, which resulted in only two networks with 10 or more members from the list of changing proteins (Supporting Information Figure 3). It was noted that a considerable number of candidate effectors from the current study, which were missing in the previous QTOF analysis, were assigned into networks that participate in protein synthesis, indicating gene translation is quickly activated in response to EphB receptor activation. Another novel group of networks are involved in cell death and growth/proliferation, which is in line with the emerging discovery that Eph receptors play important roles in cancer.[1] Compared to the QTOF study, the much improved pY proteome coverage by the Orbitrap analysis greatly facilitated pathway analysis of EphB signaling.

Conclusions

We have used SILAC and LTQ-Orbitrap mass spectrometry to screen for novel effector proteins in the EphB signaling pathway. A considerable proportion of the tyrosine phosphoproteome was identified and quantified, allowing for a global view of the changes of a huge signaling network in response to EphB receptor activation. This study revealed an unprecedented large number of candidate effectors, which will greatly accelerate the achievement of our goal of a more complete understanding of the EphB signaling pathway.

Abbreviations: IP, immunoprecipitation; pY, phosphotyrosine; SILAC, stable isotope labeling with amino acids in cell culture; RTK, receptor tyrosine kinase; GO, gene ontology; IPI, international protein index; QTOF, quadrupole time-of-flight; LC, liquid chromatography; MS, mass spectrometry; MS/MS, tandem mass spectrometry; HRP, horseradish peroxidase.

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Supporting Information Available: Supporting Information Table 1 shows SILAC ratios and Pfam domain annotations for identified proteins. Supporting Information Table 2 shows the identified phosphopeptides and localization of phosphorylation sites using Ascore. Supporting Information Table 3 shows proteins that showed significantly different SILAC ratios in this study and a previous study that used QTOF mass spectrometry. Supporting Information Table 4 contains a list of all proteins with SILAC ratios showing more that 1.5-fold change after ephrin stimulation from both replicates of the Orbitrap analysis (reported here) as well as those found in our previous QTOF study. Supporting Information Table 5 shows known effector proteins in EphB signaling. Supporting Information Figure 1 shows SILAC ratios measured by the QTOF and the Orbitrap of the proteins that were identified in both studies. Supporting Information Figures 2 and 3 show the protein networks from the Ingenuity Pathways Analysis software. Supporting Information File1 and File2 show the annotated MS/MS spectra of identified phosphopeptides. This material is available free of charge via the Internet at http://pubs.acs.org.

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