RESEARCH ARTICLE

QIKS – Quantitative identification of kinase substrates

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Signaling networks regulate cellular responses to external stimuli through post-translational modifications such as protein phosphorylation. Phosphoproteomics facilitate the large-scale identification of kinase substrates. Yet, the characterization of critical connections within these networks and the identification of respective kinases remain the major analytical challenge. To address this problem, we present a novel approach for the identification of direct kinase substrates using chemical genetics in combination with quantitative phosphoproteomics. Quantitative identification of kinase substrates (QIKS) is a novel-screening platform developed for the proteome-wide substrate-analysis of specific kinases. Here, we aimed to identify substrates of mitogen-activated protein kinase/Erk kinase (Mek1), an essential kinase in the mitogen-activated protein kinase cascade. An ATP analog-sensitive mutant of Mek1 (Mek1-as) was incubated with a cell extract from Mek1 deficient cells. Phosphorylated proteins were analyzed by LC-MS/MS of IMAC-enriched phosphopeptides, labeled differentially for relative quantification. The identification of extracellular regulated kinase 1/2 as the sole cytoplasmic substrates of MEK1 validates the applicability of this approach and suggests that QIKS could be used to identify substrates of a wide variety of kinases.

Keywords:
Cell biology / Chemical genetics / Kinase substrate identification / Mitogen-activated protein kinase / Phosphoproteomics / Quantitative proteomics

1 Introduction

Protein phosphorylation is a key mechanism of signal transduction and regulates proliferation, differentiation, survival and migration. Often, the deregulation of these signaling networks is associated with key hallmarks of cancer. The fundamental understanding of signal transduction at the molecular level requires the detailed analysis of involved phosphorylated proteins. Phosphoproteomic technologies allow the identification of thousands of phosphopeptides in a single experiment [1–3]. Yet, the functional significance of many phosphorylation sites remains unclear. For the majority of the identified phosphoproteins, critical

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Abbreviations: Erk1/2, extracellular regulated kinase 1/2; **MAPK,** mitogen-activated protein kinase; **MB-ATP,** N^6(2-methylbutyl)-ATP; **MEF,** mouse embryonic fibroblast; **Mek1,** mitogen-activated protein kinase/Erk kinase; **Mek1-as,** analog-sensitive Mek1; **QIKS,** quantitative identification of kinase substrates

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network connections have not been analyzed. Moreover, the identification of direct kinase targets is lacking. Hence the analysis and the identification of specific kinase substrates represent a major challenge in the study of signaling events.

Therefore, we developed a novel combination of biochemical strategies, chemical genetics and quantitative phosphoproteomics methods for the analysis of direct kinase substrates on a proteome wide scale. This new workflow was designed to ensure kinase specificity as well as quantitative evaluation and identification of direct substrate proteins. We propose to call this method QIKS (quantitative identification of kinase substrates). To validate QIKS, we attempted to analyze the substrate proteins of mitogen-activated protein kinase/Erk kinase (Mek1), an essential kinase of the well-characterized mitogen-activated protein kinase (MAPK) cascade. The MAPK signaling pathway regulates many cellular events including proliferation, differentiation and migration. The core unit of the pathway is a three-kinase module: Raf, a MAP3K, phosphorylates the homolog MAP2Ks Mek1/2, which in turn activate extracellular regulated kinase 1 (Erk1) and Erk2 (also known as MAPK3 and MAPK1, respectively). The MAPKs are capable of activating multiple target proteins in various cellular compartments [4]. Signal transduction through the kinase cascade is initiated by the activation of Raf by Ras, a small G-protein activated by many cellular surface receptors, *e.g.* the epidermal growth factor receptor. Both Mek isoforms can be activated by all forms of Raf proteins, and they share Erk1 and Erk2 as their so far only known target proteins [5, 6].

2 Materials and methods

2.1 Cytosolic extracts

Confluent Mek1 deficient mouse embryonic fibroblasts (MEFs) [7, 8] were starved overnight in high glucose DMEM without FCS, harvested and homogenized. The detailed protocol is described in Supporting Information *Methods*. Cytosol was dialyzed against 40 mM TrisHCl (pH 7), 0.1% β-mercapto-ethanol, 0.1 mM EGTA and 0.1% Triton-X-100 to remove ATP, followed by an incubation at 30°C for 20 min, supplemented with protease inhibitors (10 μg/mL leupeptin, 10 μg/mL aprotinin and 1 mM PMSF) to promote dephosphorylation of proteins [9].

2.2 Mutagenesis of an ATP analog-sensitive mutant of Mek1 (Mek1-as)

Based on constitutive active rat $Mek1_{S218D/S222D}$ ($Mek1_{SD}$) cDNA (Upstate Biotechnology) as template, the primers Mek1_MG5′ (ATCTGCGGCGAGCACATGGAT)/Mek1_XhoI (CTCGAGTCAGATGCTGGCAG) and BamHI_MEK1 (GGATCCATGCCCAAGAAGAAG)/Mek1_MG3′ (ATCCATGTGCTCGCCGCAGAT) were used to change methionine 143 to glycine by overlap extension PCR. PCR-fragments were amplified to full-length $Mek1_{SDM143G}$ ($Mek1_{SDMG}$) cDNA by using the primers BamHI_MEK1 and Mek1_XhoI. Mek1 constructs were cloned into pGEX-4T (Amersham Biosciences).

2.3 Purification of GST fusion proteins

Vectors for GST tagged Mek1 constructs were transformed into BL21(DE3), *Escherichia coli* and purified by affinity chromatography using Glutathione-Sepharose (Amersham Biosciences) [10].

2.4 *In vitro* phosphorylation reactions

Testing N_6-modified ATP-analogs: reaction mixtures contained GST-Mek1 mutants (0.6 μM), GST-Erk2 (0.7 μM), kinase reaction buffer (50 mM HEPES (pH 7.4), 10 mM MgCl, 1 mM DTT) and 100 μM ATP or ATP-analogs.

Identification of direct kinase targets: pretreated cytosol (final concentration: 0.7 μg/μL) was incubated with GST-$Mek1_{SDM142G}$ (0.6 μM) in the presence of 250 μM N^6(2-methyl-butyl)-ATP (MB-ATP) in kinase reaction buffer containing 2 mM Na_3VO_4, 50 mM NaF, 20 mM β-glycerophosphate, 10 μg/mL leupeptin, 1 μg/mL pepstatin, 10 μg/mL aprotinin and 0.4 mM pefablocSC.

Phosphorylation reactions were incubated for 20 min at 30°C and terminated by protein precipitation [11]. For Western blotting, proteins were dissolved in 60 mM TrisHCl (pH 6.8), 11% glycerol, 2% SDS, 0.01% bromophenol blue and 100 mM DTE. For peptide-IMAC protein pellets were washed three times in MeOH to remove traces of ATP-analog and dissolved in 8 M urea, 0.5 M ammonium bicarbonate to a final concentration of 2 mg/mL.

2.5 Tryptic digest and methylester-modification

Proteins were digested with Lys-C and trypsin as described in Supporting Information *Methods*. Peptides were desalted using StrataX 33 μm columns (Phenomenex) and eluted in 300 μL 70% ACN/1% TFA. For the methylester modification reaction, 500 μL normal or deuterated MeOH were mixed carefully with 80 μL acetylchloride (anhydrous) on a cooling bath. Three hundred and fifty microgram of the PLUS and 350 μg of the MINUS sample were split into two parts of 175 μg. One part of the PLUS sample was labeled with 200 μL of light methanolic HCl and the other part with 200 μL of heavy methanolic HCl, and the MINUS sample was treated identically. After 2 h incubation (RT) the samples were lyophilized overnight. The four dried pellets (PLUS-light, PLUS-heavy, MINUS-light, MINUS-heavy) were dissolved in 75 μL of 30% ACN/30% MeOH/40% H_2O.

Seventy-five microliter of the heavy labeled sample (PLUS) was mixed with 75 μL of the light labeled sample (MINUS) and *vice versa*, corresponding to the two technical replicates.

2.6 IMAC

Two microliter PhosSelect Iron Affinity gel (Sigma), washed with 90 μL ACN/MeOH/HAc, were incubated with 350 μg of protein digest under rotation for 45 min and filled into compressed gel loader tips. Non-phosphorylated proteins were removed by washing with 3 × 20 μL HAc, pH4 (15 μL of a 10% solution of HAc in 50 mL H_2O), phosphopeptides were eluted in two steps with 40 μL of 50 μmM Na-phosphate, pH 6, followed by 40 μL of 125 μmM Na-phosphate, pH 6.

2.7 MS data analysis

Acquired data (Xcalibur RAW-file) were converted into Sequest DTA-files using Bioworks Browser 3.2. (Thermo Electron). DTA-files were searched against the nr-(ftp://ftp.ncbi.nlm.nih.gov/blast/db/FASTA/nr.gz), version 2006/12/22, or ipi-database (ftp://ftp.ebi.ac.uk/pub/databases/IPI/current/ipi.MOUSE.fasta.gz), version 2006/12/22, selected for mouse-proteins using Sequest Bioworks Browser. Search for serine-phosphorylated peptides was repeated using the ipi.MOUSE-database version 2009/12/19 containing 113 662 sequences and 50 996 330 residues. Search parameters are listed in Supporting Information *Methods*.

2.8 Relative quantification of phosphopeptides

Results from database searches were filtered for SEQUEST scores (XCorr of peptides (1^+, 2^+, 3^+: 1.8, 2.2, 3.3), Sf>0.40, peptide mass accuracy 5 ppm, percent ions>15) to contain less than 1% false-positive protein identifications. The rate of false-positives was calculated by performing SEQUEST database searches against a target/decoy database consisting of a combined forward and reverse version of the IPI mouse database [12, 13]. All phosphopeptides positively identified in at least two experiments were quantified using the Sequest PepQuan tool applying the following parameters: mass tolerance 0.05, minimum threshold 0.05, number of smoothing points 13. The calculated regulatory ratio of all corresponding heavy and light peptide pairs were manually inspected and, if necessary, peak area integration was corrected using PepQuan. In addition, peak areas for threonine- and tyrosine-phopshoryated peptides were calculated manually in Xcalibur.raw-files using Qual Browser (Xcalibur, Thermo Fisher Scientific) using the parameters – mass tolerance: 5 ppm, minimum threshold: 500, number of smoothing points: 13. All MS/MS spectra of tyrosine- and threonine-phosphorylated peptides were validated by manual inspection. Factors such as the neutral loss of the fragment ions, the appearance of continuous ion series, the number of unexplained high-abundance peaks and the overall presence of background noise peaks were used as criteria for manual validation.

2.9 MASCOT for spectrum viewer

MS/MS spectra of quantified tyrosine- and threonine-phosphorylated peptides were searched using MASCOT 2.2.0 (Matrix Science) against the mouse KMBS database, version 2005/03/02 containing 115 660 sequences and 44 323 098 residues to generate a result format compatible with the database SpectrumDB, programmed at the IMP, described in Supporting Information *Methods*.

Additional methods including cell culture, synthesis of N^6-substituted adenosine-5′-triphosphates, antibodies for Western blotting, calculation of K_M and V_{max} for Mek1-as and nano-HPLC-MS are described in Supporting Information *Methods*.

3 Results

3.1 Experimental setup

Our goal was to establish a proteome wide screening platform that allows the faithful identification of direct kinase substrates. To validate our approach, we aimed to identify all cytoplasmic substrates of MEK1. Due to the general low abundance of signaling molecules and phosphoproteins in a complex biological sample, the detection of phosphopeptides remains challenging despite recent improvements in MS. Therefore, we reduced sample complexity at different levels of the (phospho)proteome prior to MS analysis. The key techniques of our screening platform are depicted in Fig. 1. The source of MEK1 substrates was the cytoplasmic fraction obtained from Mek1 deficient MEFs [7, 8]. Since activated Mek1 is mainly located in the cytoplasm [14], cells were fractionated and cytosolic extracts depleted from ATP. Mek1-as was generated based on the principles of chemical genetics (Fig. 1A): this method was developed by the Shokat group for the identification of direct protein kinase substrates in total cells or cell extracts [15] and has been used in variety of studies on kinase substrate phosphorylation [1]. In this approach, amino acids at the ATP-binding pocket surrounding the N^6-position of the ATP, the so-called ''gate-keeper residues,'' are exchanged to residues with smaller side chains. Thereby, the enlarged ATP-binding pocket allows the kinase to use ATP-analogs with a bulky side chain attached to the N^6-position of the adenine. Such compounds cannot be used by wild-type kinases, resulting in a decrease of background phosphorylation in ATP-depleted cell extracts and a remarkable increase in the specificity of phosphorylation reactions for the mutated kinase. Phosphorylated proteins from kinase

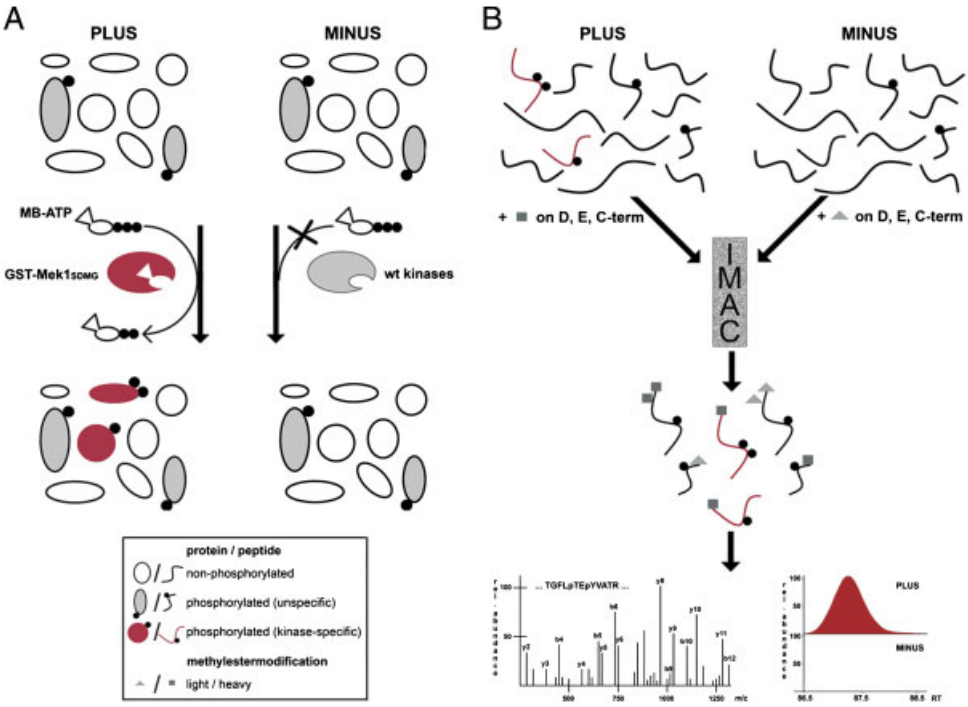

Figure 1. Schematic overview of key techniques optimized in the combinatorial screening approach for specific kinase substrates. (A) ATP-depleted cytosolic extracts of Mek1 deficient MEFs are used for *in vitro* kinase assays on the principles of chemical genetics: the structure of Mek1 (GST-Mek1$_{SDMG}$) is changed to allow the use of a bulky ATP-analog (MB-ATP), thereby increasing the specificity of the kinase reaction (PLUS) for direct Mek1 substrate proteins. Control samples (MINUS) are treated with the ATP-analog MB-ATP alone to define unspecific background phosphorylation, as wild-type kinases cannot bind the ATP-analog. (B) Samples from *in vitro* kinase reactions are submitted to tryptic digest and phosphopeptides are enriched by IMAC. *C*-termini and acidic side chains of amino acid residues (D, E) are converted to methylesters to reduce binding of non-phosphorylated peptides. Samples are labeled differentially for relative quantification by using normal (light) or deuterated (heavy) methanol in the esterification reaction. Phosphopeptide-enriched fractions are subjected to RP chromatography coupled to a LTQ-FT-ICR mass spectrometer. MS data are analyzed for corresponding phosphoproteins, and phosphorylated peptides are quantified.

assays in the presence or without Mek1-as were subjected to tryptic digest and characterized by LC-MS/MS analysis of IMAC-enriched phosphopeptides [16], which were differentially labeled for relative quantification by methylester-modifications (Fig. 1B).

3.2 Chemical genetics of MEK1

An analog-sensitive mutant of Mek1 was generated according to Cdk2-as [17] and PKA-as [15] (Fig. 2A), based on the 2.4 Å crystal structure of rat Mek1 (PDB 1s9j [18]). To generate an analog-sensitive mutant of constitutive active mutant Mek1$_{S218D/S222D}$ (Mek1$_{SD}$) [19], M143 was replaced by glycine (G), resulting in the mutant Mek1$_{SDM143G}$ (Mek1$_{SDMG}$) (Fig. 2B). The ATP-pocket mutant was expressed as GST-tagged recombinant protein in *E. coli*. The purified kinase was tested for its ability to use a selected library of N^6-modified ATP-analogs for the phosphorylation of the recombinant substrate GST-Erk2. Western blot analyses for a subset of reactions are shown in Fig. 2C. The ATP-pocket mutant GST-Mek1$_{SDMG}$ displayed the best phosphorylation efficiency with MB-ATP, while GST-Mek1$_{SD}$ with the wild-type ATP-binding pocket displayed no substrate phosphorylation with this compound (Fig. 2C). The structures of all tested ATP-analogs are shown in Supporting Information Fig. 1. Therefore, MB-ATP was our bulky ATP analog of choice for Mek1$_{SDMG}$. The relative kinetics parameters for GST-Mek1$_{SD}$ and GST-Mek1$_{SDMG}$ were determined by measuring the incorporation of ^{32}P-phosphate from γ-^{32}P-ATP into phospho-GST-Erk2. GST-Mek1$_{SDMG}$ showed a slight reduction in the relative kinase activity with a K_M of 12 μM and a V_{max} of 1.28 μM/min in comparison to GST-Mek1$_{SD}$ with 10.5 μM and 1.28 μM/min, respectively, when both kinases were used at 50 nM and GST-Erk2 at 1 μM to 16 μM. Taken together, these results showed that an analog-sensitive and functional Mek1 mutant was generated that could utilize MB-ATP as the respective bulky analog.

3.3 Phosphorylation of Mek1 substrate proteins and MS-analysis

For the identification of direct Mek1 substrate proteins, GST-Mek1$_{SDMG}$ was used for *in vitro* phosphorylation

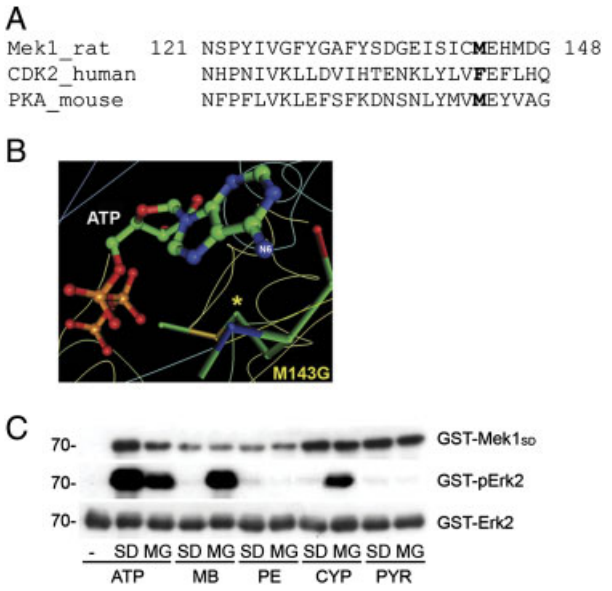

Figure 2. Mutagenesis of Mek1-as. (A) Alignment of selected regions in the Mek1, CDK2 and PKA kinase domains. The gatekeeper residue M143 in Mek1 and corresponding amino acids in CDK2 and PKA are highlighted in bold. (B) Zoom into the ATP-binding pocket of rat Mek1 with the side chain [*] of the gatekeeper residue M143 in close proximity to the N^6 position of adenine in ATP (modified from PDB 1s9j [13]). (C) Identification of ATP-analogs utilized by Mek1-as. *In vitro* kinase assays with recombinant constitutive active GST-$Mek1_{SD}$ (SD) and the ATP-pocket mutant GST-$Mek1_{SDM143G}$ (MG); GST-Erk2 was used as a substrate. Western blotting shows levels of GST-tagged kinases with anti-Mek1-specific antibodies. Substrate-phosphorylation is visualized with anti-phospho-Erk1/2-specific antibodies. Total Erk2 levels are shown with anti-Erk1/2-specific antibodies.

reactions with ATP-depleted cytosol of serum-starved $Mek1^{-/-}$ MEFs in the presence of MB-ATP (in the following referred to as PLUS-sample). A control sample without kinase (MINUS) was treated identically to measure unspecific background phosphorylation. The use of the ATP-analog reduced the background phosphorylation by wild-type kinases present in the cytosolic extract as demonstrated by Western blot analysis with anti-phosphotyrosine specific antibodies (Supporting Information Fig. 2). These reaction mixtures were subjected to tryptic digestion. Prior to IMAC enrichment of phosphorylated peptides, acidic side chains of the amino acids glutatmate (E) and aspartate (D) as well as *C*-termini of peptides were modified by methyl-esterification for two reasons: (i) esterification of acidic side chains has been shown to improve the specificity of IMAC-enrichment for phosphopeptides [16]; (ii) to quantitatively label proteins we used normal (light) or deuterated (heavy) methanol in the esterification reaction, which introduced a mass difference of 3 Da *per* modification between identical peptides derived from corresponding PLUS and MINUS samples. The labeling efficiency was similar for both esterification reagents: in the ''light'' group, 97.7% of C-termini and 97.4% of D/E amino acids were labeled. In the ''heavy'' group, the corresponding percentages were 96.7% of *C*-termini and 98.3% of D/E amino acids.

Each peptide was labeled at least once on the *C*-terminus. Therefore, esterified digests from corresponding PLUS and MINUS samples could be combined, purified by IMAC and analyzed together to avoid run-to-run variations for later quantification. Phosphopeptide-enriched fractions were subjected to reverse phase chromatography coupled to LTQ-FT-ICR MS. Samples were measured in two technical replicates: For one measurement, the PLUS sample was labeled with heavy methanol and the MINUS sample with light methanol. For the technical replicate, the two labels were exchanged, resulting in a light labeled PLUS sample and a heavy labeled MINUS sample, respectively, to control that the different labeling reagents themselves introduce no differences in the enrichment or fractionation behavior of peptides. We observed a reproducible retention time difference in RP-HPLC for deuterated (heavy) peptides when compared with their non-deuterated counterparts (light), as shown in Fig. 3C. This separation has been described for various labeling techniques using deuterated chemicals [20, 21]. On an average, 1622 phosphorylation sites on 827 phosphopeptides were identified. Overall, acquired data were in agreement with recent literature from large-scale phosphoproteomics studies. The percentage of phosphorylated amino acid residues was 93.7% for phospho-serine, 5.7% for phospho-threonine and 0.7% for phospho-tyrosine. The ratio of phosphorylation sites *per* peptide after IMAC-enrichment was 22.9% for singly phosphorylated, 57.0% for doubly phosphorylated and 20.1% for triply phosphorylated peptides, respectively. Data from the two technical replicates were highly reproducible, as shown in Supporting Information Fig. 3A–3C.

3.4 Identification of direct MEK1 substrates

For the identification of direct Mek1 substrate proteins, the identified phosphopeptides were searched for phosphoproteins exclusively identified in the samples where GST-$Mek1_{SDMG}$ was added. The only so far described Mek1 targets Erk1 and Erk2 were identified in PLUS samples, but not in MINUS samples. The known phosphorylation sites upon activation by Mek1 could be identified: T202 and Y204 for Erk1 as well as T183 and Y185 for Erk2 [22]. This finding was confirmed by Western blot analysis of PLUS and MINUS samples using antibodies specific for phosphorylated Erk1/2 (Fig. 3A). To identify novel Mek1 substrates, we focused on peptides phosphorylated on either tyrosine or threonine. We expected that Mek1 substrate proteins display similar phosphorylation motives and, therefore, should be phosphorylated on the same amino acid residues as Erk1/2. Except for Erk1 and Erk2, we were unable to identify phosphopeptides exclusively enriched in samples where GST-$Mek1_{SDMG}$ was added. In this type of analyses,

remaining background phosphorylation in the cytosolic extracts could mask putative Mek1 substrates. To overcome this problem, we used differential peptide labeling for relative quantification and searched for proteins identified in both PLUS and MINUS $Mek1_{SDMG}$ samples, but present in higher amounts where $Mek1_{SDMG}$ was added. For this purpose, threonine- or tyrosine-phosphorylated peptides identified with a high confidence were quantified in both experiments. An example for the relative quantification is demonstrated in Fig. 3B for the Mek1 substrate Erk1, exclusively identified in the PLUS sample, whereas a non-regulated peptide is present in similar amounts in PLUS and MINUS samples (Fig. 3C). Results for Erk1/2-phosphopeptides and 43 additional peptides that were quantified in both technical replicates are listed in Table 1. Ratios of peptide amounts in PLUS *versus* MINUS samples are shown in Fig. 3D. Representative MS/MS spectra for all quantified peptides are stored in SpectrumDB, a new database for

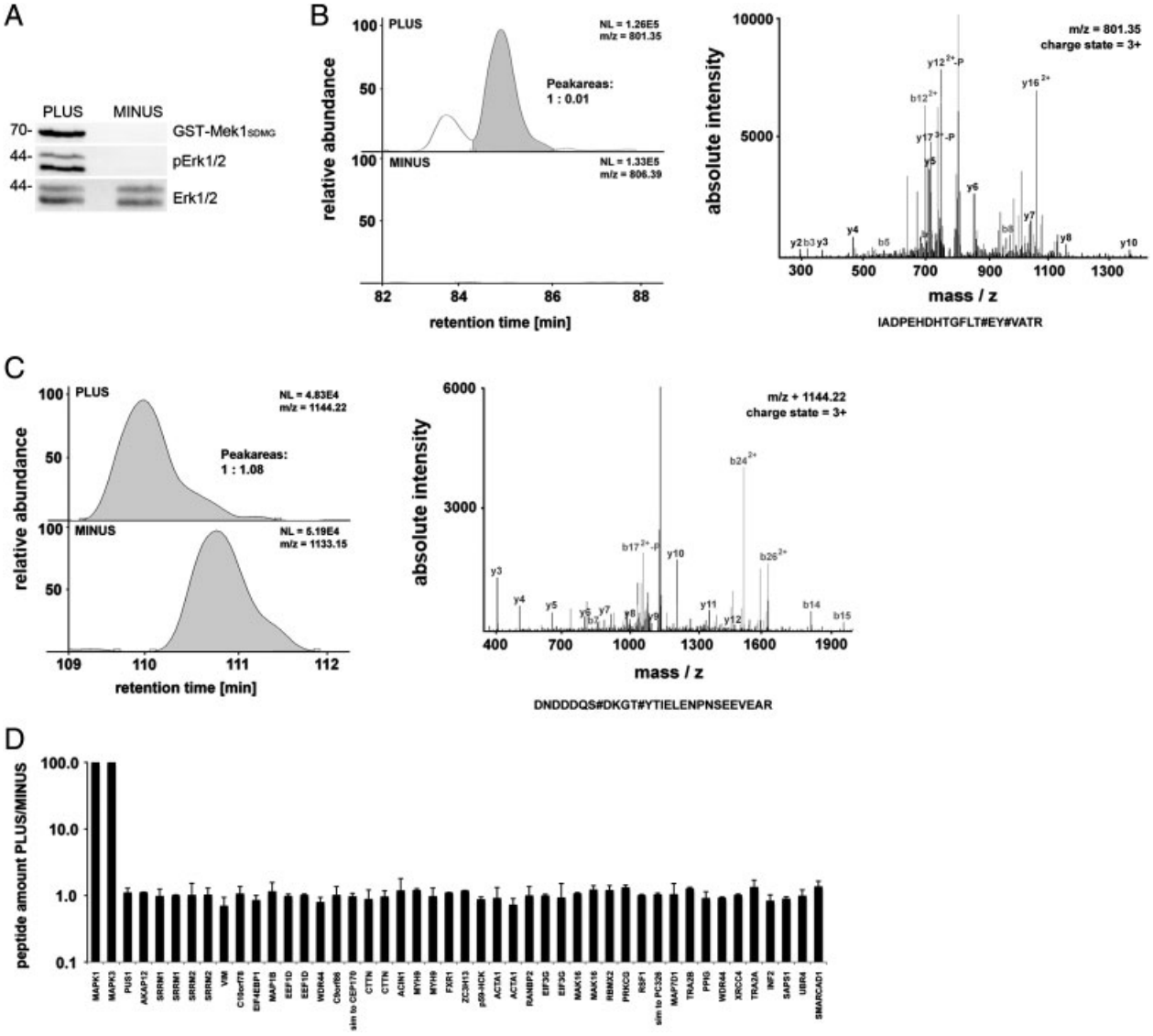

Figure 3. Identification of direct Mek1 substrates. (A) Phosphorylation of endogenous Erk1/2 upon addition of constitutive active GST-$Mek1_{SDMG}$ to cytosol of $Mek1^{-/-}$ cells in the presence of N^6(2-methylbutyl)-ATP. Western blotting shows GST-Mek1SDMG with anti-GST-specific antibodies. Phosphorylation of endogenous Erk1/2 is shown with anti-phospho-Erk1/2 specific antibodies. Levels of total Erk1/2 are visualized with anti-Erk1/2 specific antibodies. (B/C) Manual quantification of phosphopeptides; left: peak areas for corresponding peptides with normal and deuterated methylester labels; right: corresponding MS2 spectrum for peptide in PLUS. (B) Phosphopeptide IADPEHDHTGFLT♯EY♯VATR of Erk1 (MAPK3), exclusively present in PLUS where GST-$Mek1_{SDMG}$ was added. (C) Phosphopeptide DNDDDQS♯DKGT♯YTIELENPNSEEVEAR, a typical background phosphorylation present in similar amount in PLUS and MINUS samples. NL, absolute peak intensity. (D) ratio of peptide amounts in PLUS *versus* MINUS samples for quantified threonine- and tyrosine-phosphorylated peptides; the ratio of Erk1 and Erk2 peptides was set to 1:0.01 (PLUS/MINUS).

Table 1. Quantification of threonine- and tyrosine-phosphorylated peptides from *in vitro* kinase assays using cytosol of Mek1$^{-/-}$ cells and constitutive active GST-Mek1$_{SDMG}$ in the presence of N^6(2-methylbutyl)-ATP[a)]

TR1		TR2		Phosphopeptide	*m/z*	*z*	Sf	XCorr	DeltaCn	Mass error (ppm)	Mascot score	Rank	Accession number	Gene symbol	Gene name
+	−	+	−												
1	0.00	**1**	0.00	VADPDHDHTGFL**T**♯**E**Y♯VATR	797.04	3	0.89	5.08	0.21	0.0	28	1	IPI00119663.2	MAPK1	Mitogen-activated protein kinase 1
1	0.01	**1**	0.00	IADPEHDHTGFL**T**♯**E**Y♯VATR	806.39	3	0.86	5.67	0.15	1.0	44	1	IPI00230277.2	MAPK3	Mitogen-activated protein kinase 3
1	1.00	1	0.80	VPSSLEG**S**♯EGDGD**T**♯D	863.87	2	0.77	3.11	0.27	1.9	27	1	IPI00123912.4	PUS1	Pseudouridylate synthase 1
1	0.90	1	0.89	SA**T**♯**LS**♯**S**♯TESTASGMQDEVR	1132.96	2	0.79	3.96	0.00	1.0	53	1	IPI00123709.1	AKAP12	A kinase (PRKA) anchor protein 12
1	1.20	1	0.83	HRPS**S**♯PA**T**♯PPPK	483.56	3	0.49	2.79	0.01	0.2	19	1	IPI00118438.4	SRRM1	Serine/arginine repetitive matrix protein 1
1	0.98	1	1.00	KE**T**♯**ES**♯EAEDDNLDDLER	1154.48	2	0.88	3.75	0.47	0.5	52	1	IPI00118438.4	SRRM1	Serine/arginine repetitive matrix protein 1
1	1.29	1	0.67	S♯GTPPRPGSVT♯NMQADECTATPQR	920.72	3	0.57	3.73	0.01	0.9	16	1	IPI00225062.2	SRRM2	Serine/arginine repetitive matrix protein 2
1	1.14	1	0.80	SLT♯R**S**♯PPAIR	636.30	2	0.50	2.88	0.05	0.2	11	1	IPI00225062.2	SRRM2	Serine/arginine repetitive matrix protein 2
1	1.75	1	1.10	SLY♯SSSPGGAYVTR	771.36	2	0.78	2.51	0.10	−2.2	23	1	IPI00227299.5	VIM	vimentin
1	1.11	1	0.76	ENPPSPP**T**♯**S**♯PAAPQPR	612.94	3	0.51	3.58	0.16	−0.2	4	1	IPI00458153.2	C10orf78	Uncharacterized protein C10orf78 homolog
1	1.31	1	1.03	FLMECRN**S**♯PVAK**T**♯PPK	690.33	3	0.76	3.63	0.35	0.5	8	1	IPI00318938.5	EIF4EBP1	Eukaryotic translation initiation factor 4E binding protein 1
1	1.07	1	0.66	VQSLEGEKLS♯PK**S**♯DISPL**T**♯PR	863.76	3	0.76	5.06	0.07	n.i.	n.i.	1	IPI00130920.1	MAP1B	Microtubule-associated protein 1B
1	0.97	1	1.06	KGA**T**♯PAEDDEDKDIDLFG**S**♯DEEEEDK	1099.23	3	0.92	4.86	0.56	3.8	42	1	IPI00118875.3	EEF1D	Eukaryotic translation elongation factor 1 delta
1	0.96	1	1.00	GA**T**♯PAEDDEDKDIDLFG**S**♯DEEEEDK	1056.52	3	0.88	4.74	0.42	1.5	14	1	IPI00118875.3	EEF1D	Eukaryotic translation elongation factor 1 delta
1	1.40	1	1.10	EYVSNDA**T**♯Q**S**♯DDEEK	994.38	2	0.40	2.98	0.04	0.7	58	1	IPI00308560.6	WDR44	WD repeat domain 44
1	1.20	1	0.76	FPVREDL**S**♯DV**T**♯DEDTGPAQPPPPSK	984.78	3	0.61	3.36	0.11	−0.2	23	1	IPI00349069.4	C9orf86	Chromosome 9 open reading frame 86
1	1.10	1	0.95	DNDDDQS♯DKGT♯YTIELENPNSEEVEAR	1144.22	3	0.64	3.78	0.01	2.5	40	1	IPI00662263.1	sim CEP170	Similar to centrosomal protein 170kDa
1	1.40	1	0.85	KQ**T**♯PPASPSPQPIEDRPPS♯SPIYEDAAPFK	1164.91	3	0.57	3.85	0.03	0.7	33	1	IPI00118143.1	CTTN	Cortactin
1	1.19	1	0.88	KQ**T**♯PPAS♯PSPQPIEDRPPS♯SPIYEDAAPFK	1191.57	3	0.85	5.78	0.01	1.2	45	1	IPI00118143.1	CTTN	Cortactin
1	1.11	1	0.57	APVVLQPEQIV**S**♯EEE**T**♯PPPLLTK	920.50	3	0.77	4.01	0.30	0.4	24	1	IPI00121136.6	ACIN1	Apoptotic chromatin condensation inducer 1
1	0.86	1	0.80	KG**T**♯GDC**S**♯DEEVDGK	870.83	2	0.88	3.23	0.66	−0.3	35	1	IPI00123181.2	MYH9	Myosin, heavy chain 9, non-muscle
1	1.24	1	0.80	KG**T**♯GDC**S**♯DEEVDGKADGADAK	807.71	3	0.56	2.59	0.41	0.0	14	1	IPI00123181.2	MYH9	Myosin, heavy chain 9, non-muscle
1	0.91	1	0.90	DPDSNPYSLLDN**T**♯E**S**♯DQTADTDASESHHSTNR	958.94	4	0.62	3.58	0.02	2.5	20	1	IPI00122521.1	FXR1	Fragile X mental retardation, autosomal homolog 1
1	0.84	1	0.85	GPR**T**♯P**S**♯PPPPILEDIILGK	736.73	3	0.97	4.95	0.66	0.9	50	1	IPI00515528.2	ZC3H13	Zinc finger CCCH-type containing 13
1	1.20	1	1.07	IIEDNEY♯TAR	686.35	2	0.41	2.66	0.00	-0.1	23	1	IPI00129487.5	p59-HCK	Hemopoietic cell kinase isoform p59Hck

Table 1. Continued

TR1 +	TR1 −	TR2 +	TR2 −	Phosphopeptide	*m/z*	*z*	Sf	XCorr	DeltaCn	Mass error (ppm)	Mascot score	Rank	Accession number	Gene symbol	Gene name
1	1.40	1	0.78	DL**T**♯DYLMK	565.28	2	0.90	2.75	0.33	-0.3	26	1	IPI00110827.1	ACTA1	Actin, alpha 1, skeletal muscle
1	1.60	1	1.15	SY♯ELPDGQVITIGNER	970.00	2	0.80	2.87	0.00	0.8	38	1	IPI00110827.1	ACTA1	Actin, alpha 1, skeletal muscle
1	0.76	1	1.23	DDDAYK**T**♯ED**S**♯DDIHFEPVVQMPEK	1041.78	3	0.74	4.65	0.02	2.0	23	1	IPI00337844.4	RANBP2	RAN binding protein 2
1	1.03	1	0.97	GIPLPTGDT♯S♯PEPELLPGDPLPPPK	927.15	3	0.64	3.66	0.01	1.1	43	1	IPI00622371.3	EFI3G	Eukaryotic translation initiation factor 3, subunit G
1	1.48	1	0.67	GIPLPTGDT♯SPEPELLPGDPLPPPK	895.47	3	0.62	3.46	0.00	−0.5	18	1	IPI00622371.3	EFI3G	Eukaryotic translation initiation factor 3, subunit G
1	0.91	1	0.95	LN**T**♯D**S**♯EEDQDDESSNDEEAHK	907.68	3	0.67	3.86	0.32	−0.4	11	1	IPI00221690.1	MAK16	MAK16 homolog
1	0.90	1	0.73	LN**T**♯D**S**♯EEDQDDESS♯NDEEAHK	945.41	3	0.43	3.56	0.07	1.9	13	1	IPI00221690.1	MAK16	MAK16 homolog
1	0.93	1	0.73	GCGVK**T**♯PPS♯SPPEV**S**♯EDEDAK	837.33	3	0.66	4.62	0.03	1.7	17	1	IPI00261255.4	RBMX2	RNA binding motif protein, X-linked 2
1	0.80	1	0.71	**T**♯FCGTPDYIAPEIIAYQPYGK	842.73	3	0.80	3.81	0.18	0.6	38	1	IPI00122069.1	PRKCG	Protein kinase C, gamma
1	1.00	1	0.97	IET♯DEE**S**♯CDNAHGDADQPAR	842.71	3	0.73	3.76	0.28	1.6	23	1	IPI00122845.10	RSF1	Remodeling and spacing factor 1
1	0.99	1	0.93	DSALQD**T**♯DDS♯DDDPVLIPGAR	829.69	3	0.56	3.60	0.02	−0.1	32	1	IPI00120084.1	SimPC326	Similar to PC326 protein
1	1.23	1	0.68	EPAAPA**S**♯PAP**S**♯PVPSPT♯PAQPQK	832.71	3	0.47	3.49	0.07	0.3	19	1	IPI00282957.4	MAP7D1	MAP7 domain containing 1
1	0.80	1	0.76	RPH**T**♯PTPGIYMGR	526.25	3	0.58	3.37	0.21	1.1	8	2	IPI00139259.1	TRA2B	Transformer 2 beta homolog
1	1.28	1	0.91	KCDHE**S**♯**S**♯PGT♯DEDK	973.88	2	0.62	3.01	0.29	1.6	26	1	IPI00349306.1	PPIG	Peptidylprolyl isomerase G (cyclophilin G)
1	0.96	1	1.00	TPEEHGLYDGS♯T♯DEESGAPVQAAETLHK	1088.86	3	0.60	4.46	0.04	1.2	34	1	IPI00403043.1	XRCC4	X-ray repair complementing defective repair in Chinese hamster cells 4
1	0.88	1	0.61	**S**♯R**S**♯Y**T**♯PEYR	713.75	2	0.72	2.68	0.10	0.6	22	1	IPI00377298.2	TRA2A	Transformer-2 alpha
1	1.38	1	1.01	TGPGEDEDGED**T**♯APESALDT**S**♯LDR	926.38	3	0.61	3.60	0.14	0.2	24	1	IPI00387449.6	INF2	Inverted formin, FH2 and WH2 domain containing
1	1.06	1	1.16	SGG**S**♯**T**♯D**S**♯EEEDEEEDEEEDEGAEQAACGR	1204.10	3	0.94	6.42	0.00	0.4	41	1	IPI00380742.3	SAPS1	SAPS domain family, member 1
1	1.16	1	0.85	HVTLPSS♯PR**S**♯N**T**♯PMGDKDDDDDDDADEK	1156.13	3	0.50	3.82	0.00	0.2	38	1	IPI00378681.4	UBR4	Ubiquitin protein ligase E3 component n-recognin 4
1	0.83	1	0.63	AN**T**♯PD**S**♯DVTEK	702.81	2	0.53	3.24	0.15	−0.4	13	1	IPI00556837.1	SMARCAD1	SWI/SNF-related, matrix-associated actin-dependent regulator of chromatin, subfamily a, containing DEAD/H box 1

a) Results from database searches using SEQUEST algorithm were filtered according to XCorr of peptides (1+, 2+, 3+: 1.8, 2.2, 3.3), Sf>0.40, modifications, peptide mass accuracy 5 ppm, number of top matches = 1 number of percent ions 15. SEQUEST and MASCOT scores are shown for the reference spectrum in SpektrumDB. Phosphorylation sites are indicated with ♯ after the corresponding amino acid residue. Phosphosites confirmed by manual evaluation are marked in bold: phosphosites that could reside on more than one residue are underlined. Numbers indicate the ratio of peaks areas of PLUS- *versus* MINUS-sample, differences of more than 1:0.2 are highlighted in bold. Peptides quantified in both technical replicates are listed. (TR, technical replication; +, PLUS-sample; −, MINUS-sample).

proteomics data created at the Institute of Molecular Pathology (IMP), Vienna, Austria, and are accessible *via* the web-page: http://www.imp.ac.at/research/protein-chemistry/labhomepage/spectrumdb/?expname = mek1_chemical_genetics. In addition, spectra are shown as Supporting Information. All peptides phosphoryated on threonine or tyrosine, except for Erk1/2-peptides, were present in equal amounts or less than 1.8-fold up- or downregulated.

To complete the data analysis, phosphoserine containing peptides were quantified as well. No serine-phosphorylated peptide showed a reproducible up- or downregulation in the Mek-1_{SDMG} MINUS samples. Data for 501 phosphoserine peptides identified in ipi-database search are shown in Supporting Information Table 1. Overall, not a single phosphopeptide other than Erk1/2-pepides showed clear changes in abundance upon addition of $Mek1_{SDMG}$. These results strongly suggest that the only direct/most abundant Mek1 substrates in cytotol are Erk1 and Erk2.

4 Discussion

Proteomics methods are powerful tools for the characterization of phosphorylation events in complex signaling networks. However, our knowledge about the specific activation and regulation of these sites is relatively small. Based on the success of the first kinase specific drugs, the immunosuppressor and mTOR-inhibitor Rapamycin, or Gleevec, targeting Bcr-Abl in chronic myelogenous leukemia, protein kinases are now targeted in many therapeutic approaches [23, 24]. Yet, relatively little is known about the substrates they activate. Classical methods for the identification of kinase substrates are often not suitable for complex samples, mainly because of unspecific background phosphorylation caused by numerous kinases present in cell extracts. This problem was addressed in various methods for the identification of kinase targets, *e.g.* in the KESTREL approach (kinase substrate elucidation and tracking) [9]. In this screening method, cell extracts are fractionated and depleted from endogenous ATP. Kinase assays are performed with short labeling times in the presence of highly radioactive γ-^{32}P-ATP and Mn^{2+} instead of Mg^{2+}. Thus, the method is restricted to kinases that can efficiently use Mn^{2+}. Analog-sensitive kinases have been used in a variety of studies on specific kinase substrates [15], but analysis of phosphorylated proteins was mainly performed with classical methods such as incorporation of radioactivity followed by one- or two-dimensional gel electrophoresis [25, 26] or by immunoprecipitation [27] rather than with quantitative proteomics techniques. Newer methods based on affinity purification of thiophosphorylated substrates [28, 29] require a kinase to utilize ATPγS analogs, which requires specialized reagents and, thus, could preclude certain kinases. Our established screening approach combines the power of phosphoproteomics for the identification and quantification of hundreds of phosphorylation sites in a single experiment with strategies for the identification of specific kinase targets.

In our search for direct target proteins of Mek1, the known substrates Erk1/2 were identified and distinguished significantly from remaining background phosphorylation. Interestingly, we could not identify novel Mek1 substrates, which is consistent with the high specificity and selectivity reported for Mek1 in the literature [6]. This is an important finding on a proteome wide level and supports the assumption that Mek1 only phosphorylates Erk1/2. However, it could as well be that other substrates of Mek1 could not be found due to the biological setup applied. The two Mek1 substrates Erk1/2 were identified in a screen exclusively performed with cytosolic proteins. By including other subcellular compartments, such as membranes, organelles or nuclear extracts, the approach can be applied on a proteome wide level. An advantage of the here established screening method is that it can easily be adapted for different biological contexts, in case kinase-deficient cell lines are available.

Many key cellular processes such as differentiation or proliferation are the result of the coordinated action of many proteins in macromolecular assemblies. Different scaffold proteins for the MAPK pathway regulate intracellular signaling by providing spatial and temporal specificity [30]. One example is kinase suppressor of Ras, which binds Raf, Mek and Erk at the plasma membrane and has been described as a regulator of cell cycle reinitiation following DNA damage [31], cellular proliferation and transformation [32]. We have shown that the localization of another MAPK scaffold complex with Mek partner 1 (MP1 or MAPBP) and its adaptor p14 (MAPBPIP) to late endosomes is required for the regulation of late endosomal traffic and proliferation [33, 34]. Activation of endogenous Erk1/2 by Mek1 is enhanced in the presence of the scaffold complex MP1/p14 as shown by Western blotting of kinase reaction in the presence or without MP1/p14 in cell extracts from $p14^{-/-}$ MEFs [34] (Fig. 4). Scaffold complexes might

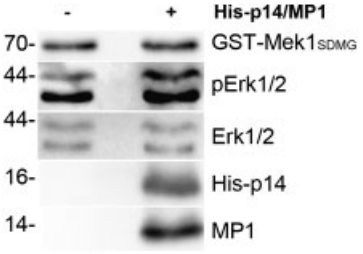

Figure 4. Enhanced activation of endogenous Erk1/2 by GST-$Mek1_{SDMG}$ in the presence of the scaffold complex p14/MP1. ATP-depleted cytosol of $p14^{-/-}$ MEFs was incubated with GST-$Mek1_{SDMG}$ in the presence of N^6(2-methylbutyl)-ATP and without (left) or with (right) recombinant complex of His-p14/Mp1. GST-$Mek1_{SDMG}$ is shown by Western blotting with anti-GST-specific antibodies, phosphorylation of endogenous Erk1/2 with anti-phospho-Erk1/2 specific antibodies. Levels of total Erk1/2 are visualized with anti-Erk1/2 specific antibodies, His-p14 with anti-His specific antibodies and MP1 with anti-MP1 specific antibodies, respectively.

provide access to specific substrate proteins. Thus, we speculate that the application of this quantitative screening approach for scaffold-mediated signaling complexes may lead to the identification of novel and complex-specific kinase substrates. Concordantly, effects on the activation efficiency between kinases and targets upon binding to the scaffold complex could be quantified on a proteome wide scale.

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