Identification of Major ERK-Related Phosphorylation Sites in Gab1

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ABSTRACT: Gab1 (Grb2-associated binder1) belongs to a family of multifunctional docking proteins that play a central role in the integration of receptor tyrosine kinase (RTK) signaling, i.e., mediating cellular growth response, transformation, and apoptosis. In addition to RTK-specific tyrosine phosphorylation, these docking proteins also can be phosphorylated on serine/threonine residues affecting signal transduction. Since serine and threonine phosphorylation are capable of modulating the initial signal one major task to elucidate signal transduction via Gab1 is to determine the exact localization of distinct phosphorylation sites. To address this question in this report we examined extracellular signal-regulated kinases 1/2 (ERK) specific serine/threonine phosphorylation of the entire Gab1 engaged in insulin signaling in more detail in vitro. To elucidate the ERK1/2-specific phosphorylation pattern of Gab1, we used phosphopeptide mapping by two-dimensional HPLC analysis. Subsequently, phosphorylated serine/threonine residues were identified by sequencing the separated phosphopeptides using matrix assisted laser desorption ionization mass spectrometry (MALDI-MS) and Edman degradation. Our results demonstrate that ERK1/2 phosphorylate Gab1 at six serine/threonine residues (T312, S381, S454, T476, S581, S597) in consensus motifs for MAP kinase phosphorylation. Serine residues S454, S581, S597, and threonine residue T476 represent nearly 80% of overall incorporated phosphate. These sites are located adjacent to *src* homology region-2 (SH2) binding motifs (YVPM-motif: Y447, Y472, Y619) specific for the phosphatidylinositol 3kinase (PI3K). The biological role of identified phosphorylation sites was proven by PI3K and Akt activity in intact cells. These data demonstrate that ERK1/2 modulate insulin action via Gab1 by targeting serine and threonine residues beside YXXM motifs. Accordingly, insulin signaling is blocked at the level of PI3K.

Grb2-associated binder1 (Gab1)[1] belongs to the family of scaffolding or multifunctional docking proteins including Grb2-associated binders (Gab1, 2, 3), drosophila daughter of sevenless (DOS), insulin receptor substrates (IRS1−6), downstream of kinases (Dok), Dok-related (Dok-R) and fibroblast growth factor receptor substrate 2 (FRS2). These proteins lack detectable catalytic activity but recruit signaling molecules containing src homology 2 (SH2) or phosphotyrosine binding (PTB) domains when they become tyrosine phosphorylated by diverse receptor tyrosine kinases (RTKs). Accordingly, these proteins play a major role in integration as well as amplification of various signaling pathways (for a recent review, see refs *1*−*4*).

Nowadays it is commonly accepted that RTK-mediated signal transduction via those docking proteins seems to be regulated by two major mechanisms. RTK-mediated phosphorylation of specific tyrosine residues in docking proteins leads to selective recruitment of postponed signaling proteins thereby initializing cellular responses. In contrast to that, serine and threonine phosphorylation of docking proteins by a wide variety of protein kinases, i.e., PKCs (*5*−*7*), ERKs (*8*), and c-Jun N-terminal kinase (*9*), modulate the initial RTK signal, e.g., inhibiting IRS1 association to the IR (*10*, *11*) or phosphorylation of IRS1 on tyrosine residues. Furthermore, phosphorylation of serine residues in IRS1, adjacent to YXXM binding motifs for phosphatidylinositol 3-kinase (PI3K), disturbs specifically IRS1/PI3K interaction and deactivates PI3K signaling (*12*). In contrast to that, also positive regulation of docking proteins by serine/threonine phosphorylation, e.g., through inhibition of tyrosine phosphatases, has been reported (*13*).

Phosphorylation of Gab1 in response to a wide variety of growth factors, cytokines as well as B- and T-cell antigens induces interaction with multiple downstream signaling elements possessing SH2 domains in various cell systems, such as the regulatory subunit (p85) of the PI3K, phospholipase Cγ (PLC-γ), the protein tyrosine phosphatase SHP2, and Crk (*14*, *15*). Previously, we have shown that Gab1 is specifically phosphorylated by the insulin and EGF receptor

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[1] Abbreviations: ERK, extracellular signal-regulated kinase; GST, glutathione *S*-transferase; HPLC, high performance liquid chromatography; IR, insulin receptor; IRS, insulin receptor substrate; MALDI-MS, matrix assisted laser desorption ionization mass spectrometry; PI3K, phosphatidylinositol 3kinase; RTKs, receptor tyrosine kinases; SH2, src homology domain 2.

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on a common cohort of tyrosine residues but with completely different efficiencies indicating a molecular mechanism for generating signaling selectivity (*16*, *17*).

In addition to its well-characterized tyrosine phosphorylation, Gab1 exhibits 47 potential serine/threonine phosphorylation displaying consensus motifs for a wide spectrum of protein kinases including protein kinase (PK) A, PKC, PKB, and the MAP kinases (*15*). Recently, it has been shown that serine/threonine phosphorylation of Gab1 is capable of modulating the interaction and activation of PI3K induced by EGF and HGF (*18*, *19*). To elucidate the complex sequences of signal transduction via Gab1, the exact localization of phosphorylation sites targeted by specific kinases and quantifying distribution of phosphate incorporations describes a major task. In this report, we have identified serine/threonine phosphorylation sites in human Gab1 related to ERK1/2, in vitro.

EXPERIMENTAL PROCEDURES

Construction and Expression of Human Recombinant Gab1 Protein. The entire recombinant Grb2-associated binder1 (Gab1) (aa 1−724 with apparent M_r = 105.6 kDa; according to the 10 exon containing full-length human sequence (NCBI, Locus ID 2549, 154513−154642 kb)) was prepared as described previously (*16*). For transfection assays, the coding sequence of Gab1 was cloned into the pCMV-Tag 2B (Stratagene) expression vector. Expression vector pUSE/MEK2D for dominant-active MEK2 S222D/S226D under control of CMV promoter was purchased from Upstate Biotechnology.

Site-Directed Mutagenesis. The multiple Gab1 mutant Gab1-ΔP (S_{454} → A, T_{476} → V, S_{581} → A, S_{597} → A) was generated by site-directed mutagenesis using the Quick Change kit (Stratagene) according to the manufacturer's recommendations. The following primers were used: S_{454} → A (5′- GAATCC CAATGCACCACCACGAC); T_{476} → V (5′-GTGCCAATGGTTCCAGGAA C); S_{581} → A (5′-CCAGTTAGAGCTCCCATCACTAG); S_{597} → A (5′-GGTTTCCCATGGCCCCCCGACCAG).

Cell Culture, Transient Transfection, and Insulin Stimulation. CHO cells (overexpressing human insulin receptor) were maintained in DMEM (Sigma-Aldrich) supplemented with 10% (v/v) fetal calf serum (FCS) (GibcoBRL) and antibiotics (GibcoBRL) in a humidified 5% CO_2 atmosphere at 37 °C. Before transfection, cells were released by trypsinization, washed with 1× PBS, and resuspended in Opti-MEM (GibcoBRL) supplemented with 10% (v/v) FCS. For analyzing serine/threonine phosphorylation of Gab1 a cell suspension (1×10^6 cells/well) was mixed with plasmids coding for Gab1 as well as Gab1-ΔP (pCMV-Tag2B-Gab1; 2.5 μg/well) and MEK2 (pUSE/MEK2D; 1.25 μg/well) or pUSE vector (1.25 μg/well) as indicated in the legends to Figures 1 and 5. Thereafter, samples were transferred to an electroporation cuvette (inter electrode distance: 0.4 cm, BioRad) and pulsed for 18 ms in GenePulser II (BioRad). Before seeding on six-well plates (Greiner bio-one) cell suspension was diluted with DMEM supplemented with 10% (v/v) FCS and antibiotics. On day 1 after transfection cells were cultured in DMEM for 16 h. After incubating cells for 5 min without or with insulin (1×10^7 M) whole cell extracts were subjected to Western blot analyses and PI3K activity assay.

Preparation of Cell Extracts. Whole cell extracts were prepared by scraping off the cells in 300 μL of lysis buffer (50 mM Hepes, pH 7.5, 1% (v/v) Nonidet P-40, 150 mM NaCl, 5 mM EDTA, 1% (v/v) phosphatase inhibitor cocktail I and II (Sigma) supplemented with Complete (Roche)) on culture dishes after quick-freezing using liquid nitrogen. The suspensions were mixed with 20 μL of Molecular Grinding Resin (GenoTech, USA) and lysed using a disposable pestle. Subsequently lysates were centrifuged at 16000*g* for 30 min and aliquots of the supernatant were frozen in liquid nitrogen and stored at −80 °C. Protein concentration were measured using a BioRad protein assay.

PI3K Activity Assay. A total of 200 μg of protein of CHO lysates were incubated for 30 min (4 °C) with Fast Flow sepharose beads (20 μL of a 50% (v/v) slurry; Amersham Biosciences) on a rotator (60 rpm). Subsequently, the supernatant was reincubated with Red ANTI-Flag agarose beads (20 μL of a 50% (v/v) slurry; Sigma) for 2 h to immunoprecipitate overexpressed recombinant Gab1 proteins due to its N-terminal FLAG-tag. Agarose pellets were washed three times with 1 mL of lysis buffer and twice with 1 mL of assay buffer (30 mM Hepes, pH 7.5, 150 mM NaCl, 5 mM MgCl). One-half of the agarose pellet was immediately subjected to Western blot analyses using antibody against p85 subunit of PI3K (see below). Then 50 μL of assay buffer supplemented with 500 nM ATP and 50 μg/mL L-α-phosphatidylinositol (Sigma) was added to the remaining half of the agarose pellet and PI3K substrate phosphorylation was proceeded for 45 min (25 °C). PI3K activity was determined by measuring ATP consumption in the phosphorylation reaction (50 μL/probe) using Kinase-Glo Luminescent kinase assay (Promega). Luminescence measurement was performed according to the manufacturer's instructions (Promega) with a microplate luminometer LB96V (EG&G Berthold). The period of measurement was set to 10 s. To demonstrate differences in PI3K activity, basal levels of kinase activity (without insulin) were set to 1. Activity values of corresponding insulin treated samples were calculated as x-fold values.

Western Blot Analysis. SDS−PAGE was performed according to Laemmli (*20*) followed by electroblotting (*21*) to PVDF membranes (BioRad) for probing with antibodies detected by ECL (AmershamBiosciences, Uppsala, Sweden). Immunoprecipitates as well as whole cell extracts were mixed with 5× SDS loading buffer and separated by 10% SDS−PAGE. Subsequently, proteins were electroblotted to nitrocellulose membrane (Hybond ECL, Amersham Biosiences). After blocking overnight at 4 °C (1% (w/v) blocking solution (Roche) in 1× TBS, pH 7.4, membrane with proteins of immunoprecipitates or cell extracts respectively were probed with the following primary antibodies: anti-PI3K (subjunit p85; Santa Cruz, Heidelberg, Germany), anti-pY monoclonal (Y99; Santa Cruz, Heidelberg, Germany), anti-Akt, anti-phospho Akt (Cell Signaling Technology, INC; Beverly, USA) and anti-Gab1 (Upstate Biotechnology, Biozol, Eching, Germany). Kodak Xomat AR film was used for detection of chemiluminescence signals evoked by horseradish peroxidase conjugated secondary antibodies (Roche, Basel, Switzerland). Before reprobing blots were incubated with stripping solution (100 mM 2-mercaptoethanol, 2% SDS, TBS) at 55 °C for 60 min. The washed blots were reblocked prior to reprobing.

Substrate Phosphorylation Assay. Phosphorylation of GST fusion protein was performed with recombinant activated MAP kinase (20 ng of ERK1/μg of probe, 3 ng of ERK2/μg of probe) as well as recombinant soluble insulin-receptor kinase (6 ng of insulin receptor kinase/μg of probe) under conditions according to the suppliers instruction manual (Calbiochem-Novabiochem, Bad Soden, Germany), with exceptions using 25 mM Hepes/NaOH, pH 7.5, 25 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM dithiotreitol, 100 mM NaCl, 5 mM $MnCl_2$, 5 mM $MgCl_2$, 1 mM polylysine. The reaction was started by addition of ATP and [γ-^{32}P]ATP to a final concentration of 250 μM and 0.1 mCi/mL, respectively, and proceeded for 30 min at 30 °C. The reaction was terminated by addition of 2× sample buffer (50 mM Tris/HCl, pH 6.8, 100 mM DTT, 10% glycerol, 2% SDS, 0.1% bromphenol blue) as well as boiling for 5 min. Phosphorylated proteins were separated by 10% SDS−PAGE (*19*) and analyzed by autoradiography of the Coomassie-stained and dried gels. Subsequently counting Cerenkov radiation of gel slices determined phosphate incorporation.

Identification of Phosphorylation Sites by High Performance Liquid Chromatography (HPLC) and Matrix Assisted Laser Desorption Ionization Mass Spectrometry (MALDI-MS). A total of 720 μg of Gab1 fusion protein was phosphorylated by 10 μg of ERK2 under conditions as described above. The proteins were separated by SDS−PAGE, and phosphorylated Gab1 was digested with 50 μg of trypsin (sequencing grade, Roche, Basel, Switzerland) in the excised gel pieces overnight at 37 °C (*22*, *23*). The peptides were eluted with 50 mM ammoniumcarbonate and separated on an anion exchange column (Nucleogel SAX 1000−8/46, 50 × 4.6 mm, Machery & Nagel) using a Beckmann gold solvent delivery system. The HPLC flow rate was 0.5 mL/min. After injection of 1 mL of sample the peptides were eluted beginning with 100% buffer A (20 mM NH_4CH_2COOH pH 7.0) and 0% of buffer B (0.5 M KH_2PO_4 pH 4.0). Ratios of B/A were increased to 10% in 40 min and from 10 to 50% in 75 min. Fractions of 0.5 mL were collected. Radioactive fractions were subjected to reversed phase HPLC. These peptides were separated on C_{18}-reversed phase column (150 × 0.8 mm, 5 μm particle size, 300 Å pore size, LC Packings, The Netherlands) using an ABI 140 D solvent delivery system (Applied Biosystems, USA). The HPLC flow rate was adjusted to 15 μL/min via a titanium t-piece. After application of 100 μL of sample, elution started with 95% of solution A (0.1% trifluroacetic acid) and 5% of solution B (acetonitrile/water/trifluroacetic acid (84/16/0.1; v/v/v)). The concentration of solution B was raised to 50% in 90 min and from 50 to 100% in 15 min. Aliquots of the collected fractions were taken for measuring the radioactivity. Fractions containing radiolabeled peptides were subjected to Edman degradation with an Applied Biosystems model 494 cLc pulse-liquid sequenator (*24*) as well as MALDI-TOF (time-of-flight) mass spectrometry, which was performed on a REFLEX III system (Bruker Daltonik, Bremen) equipped with a SCOUT 384 multiprobe ion source. For MALDI mass spectrometry 0.5 μL of each selected fraction were directly prepared onto the MALDI target with a saturated solution of α-cyano-4-hydroxy-cinnamic acid in acetonitrile/0.1% trifluroacetic acid (1:1). Positive ion mass spectra were acquired in a linear and reflector mode using an acceleration voltage of 20000 V. In addition to manual interpretation of PSD (post source decay) fragment ion spectra the SEQUEST algorithm (OWL sequence database; University of Leeds) was used to identify the peptide phosphorylation sites (*25*).

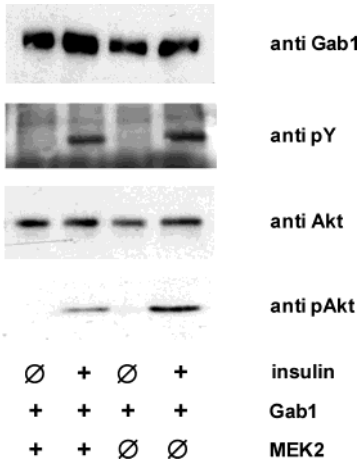


FIGURE 1: Influence of ERK on insulin-mediated Akt activation via Gab1. Relevance of ERK-specific serine/threonine phosphorylation of Gab1 for Akt activation. CHO cells overexpressing the human insulin receptor were transient-transfected with Gab1 or Gab1 and constitutive active MEK2. Lines below describe the absence (∅) or presence (+) of named substances. Aliquots of whole cell lysates were separated by SDS−PAGE and transferred to PVDF membranes (see Experimental Procedures). Blots were initially probed with anti-phospho-Akt (1:1000) followed by chemiluminescence detection. To prove equivalent protein loading blots were probed with anti-Akt (1:1000) as well as anti-Gab1 (1:1000) and with anti-pY (1:2000) to demonstrate phosphorylation of Gab1 on tyrosine residues, respectively.

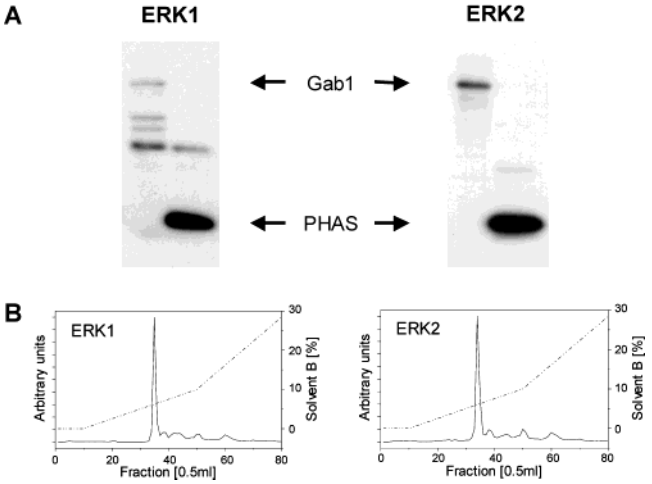


FIGURE 2: Phosphorylation of Gab1 by ERK1/2 in vitro. (A) Gab1 fusion protein (5 μg) as well as the MAP kinase substrate PHAS (2 μg; Stratagene, Heidelberg, Germany) were phosphorylated by activated MAP kinases (ERK1: 100 ng or ERK2: 15 ng) for 30 min at 30 °C as described under Experimental Procedures. Subsequently, proteins were separated by 10% SDS−PAGE, and phosphorylated proteins were visualized by autoradiography. (B) Radioactive labeled Gab1 (Figure 1A) was excised from the gel and digested in gel with trypsin. Peptides were eluted and analyzed by HPLC anion exchange chromatography. For further details, see Experimental Procedures. The gradient corresponding to the elution buffer is shown in the dashed-dotted line.

RESULTS

Recently, it has been shown that Gab1/PI3K interaction mediated by tyrosine phosphorylation through RTKs is also

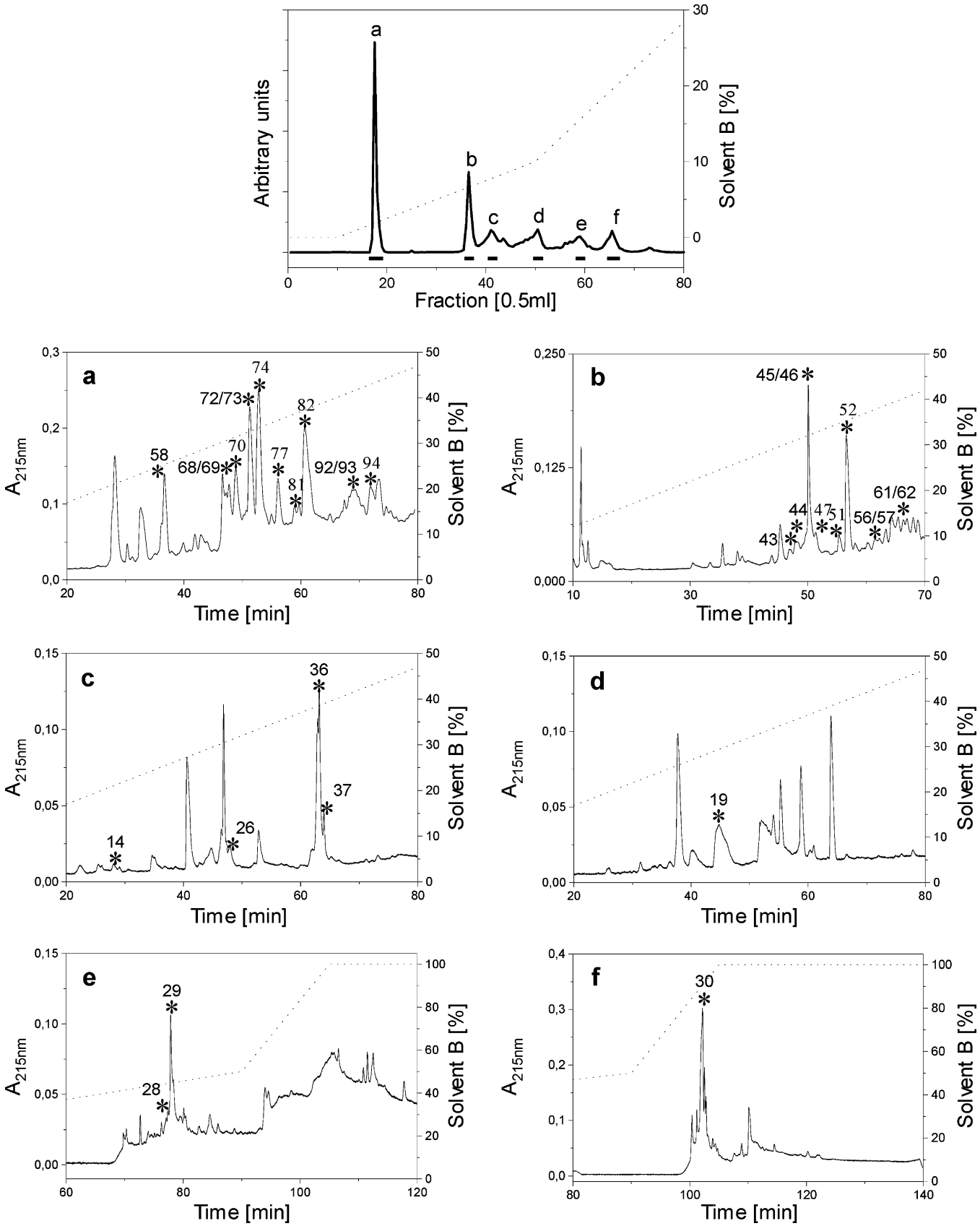


FIGURE 3: HPLC analysis of tryptic phosphopeptides derived from Gab1 phosphorylated by ERK2. A total of 730 μg of Gab1 was phosphorylated for 30 min using 10 μg of ERK2 according to Experimental Procedures. Proteins were separated by SDS−PAGE, digested with trypsin, and separated by anion exchange HPLC. Radioactive fractions were pooled according to the solid bars. Pooled fractions a−f were subjected for rechromatography to C_{18} reversed phase HPLC. Radioactive fractions are designated with asterisks followed by the fraction number.

regulated at a second level by specific phosphorylation of Gab1 on serine/threonine residues via ERK kinases. MAPK activation in RTK-selective signal transduction pathways results in divergent effects on Gab1/PI3K signaling. In HGF

signaling, ERK activation leads to an increased Gab1/PI3K interaction (*18*), whereas in EGF signaling Gab1/PI3K association is decreased (*19*).

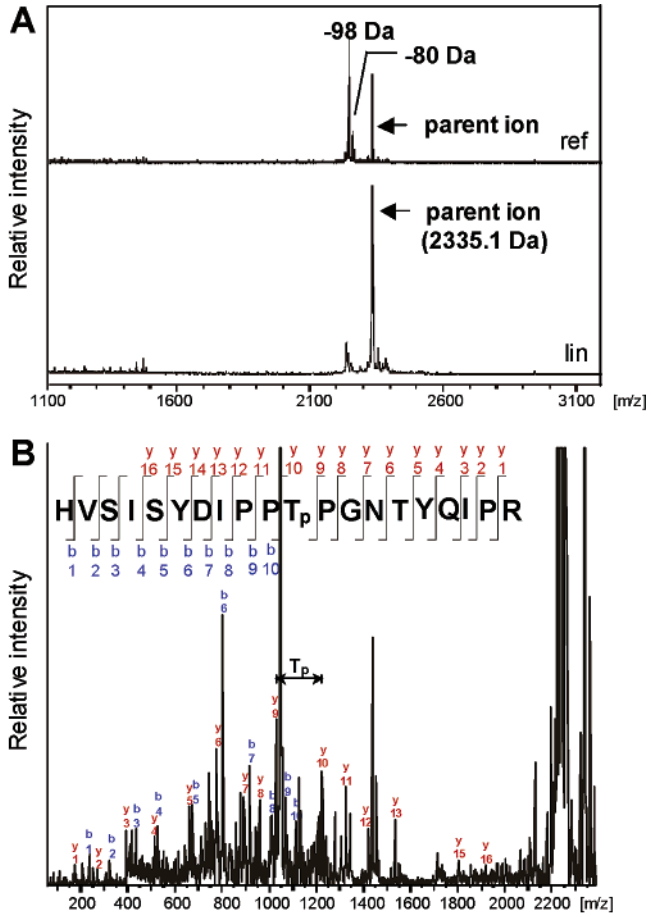
Modulation of Insulin Signaling by ERK1/2 (p44/p42 MAPK). In the case of insulin signaling via Gab1, we determined that specific activation of ERK kinases leads to a substantial decrease of phosphorylated Akt in a CHO cell system overexpressing human insulin receptor indicating less PI3K activation (Figure 1). Induction of CHO cells overexpressing the entire Gab1 with insulin resulted in elevated levels of phospho-Akt. In contrast to that, levels of phospho-Akt were significantly reduced when Gab1 was also phosphorylated by ERK1/2, which did not affect tyrosine phosphorylation of Gab1.

Peptide Map Analysis of ERK1/2 Phosphorylated Gab1. Since serine and threonine phosphorylation can modulate the initial signal, we wanted to target the ERK-related phosphorylation sites. Therefore, in a first step the entire Gab1 was expressed as glutathione-*S*-transferase (GST) fusion protein as described previously (*16*). Substrate phosphorylation of Gab1 was performed using recombinant ERK1/2. In vitro phosphorylation assays revealed that Gab1 can be substantially phosphorylated by ERK1 as well as by ERK2 (Figure 2A). To characterize whether ERK1 and ERK2 target the same phosphorylation sites in Gab1, we separated [γ-^{32}P]-ATP labeled tryptic peptides of Gab1 (analytical approach: 5 μg) using anion exchange HPLC. The radioactivity elution profile was almost identical for both MAP kinases (Figure 1B) and independent of reaction time up to 60 min (data not shown).

Identification of ERK-Specific Serine/Threonine Phosphorylation Sites in Gab1. Gab1 contains 17 potential phosphorylation sites for MAP kinases. Phosphoamino acid analyses of ERK1/2-phosphorylated Gab1 revealed phosphorylation on both serine and threonine residues (data not shown). Due to identical phosphorylation characteristics of ERK1 and ERK2 (Figure 1B) all further experiments were performed using only ERK2-phosphorylated Gab1. To identify ERK-specific S/T phosphorylation sites of Gab1 in vitro, we used a two-dimensional HPLC approach to separate in gel digested phosphorylated proteins followed by MALDI mass spectrometry and Edman degradation.

In the first dimension, tryptic peptides obtained from ERK2 phosphorylated [^{32}P]Gab1 (preparative approach: 720 μg) were separated by anion exchange HPLC. About 90% of utilized radioactivity was recovered from the gel after digestion and between 80 and 90% of the radioactive peptides were eluted from the column. This radioactivity (Cerenkov) elution profile (Figure 3) displayed one reproducible major peak (a) and five smaller peaks (b−f). To separate comigrating peptides, radioactive fractions of each peak were pooled according to the elution profile and subjected in a second dimension to C_{18} reversed phase (RP)-HPLC.

To identify peptides phosphorylated on S/T residues these radiolabeled RP-HPLC fractions were measured in the linear mode as well as in the reflector mode of a MALDI-MS instrument. Therefore, the loss of phosphate marker ions ([M+H]$^+$-80 Da (loss of HPO_3^{2-}) and [M+H]$^+$-98 Da (loss of $H_3PO_4^-$)), which is only observed in the reflector mode but not in the linear mode (e.g., Figure 4A) of a MALDI-MS instrument, indicates the existence of a phospho-S/T. For example, the spectrum derived from RP-HPLC fraction



SEQUEST v.27 (rev.11), © 1993 - 2000, Molecular Biotechnology, Univ. of Washington, J. Eng/J.Yates, Licensed for ThermoQuest Corp.
03/28/2000, (M+H)$^+$ mass = 2335.1001 ~ 0.5000 (+1), *fragment tol* = 1.3, MONO/MONO, *total inten* = 6139.5, *lowest Sp* = 0.0, matched peptides = 248, # proteins = 312944, Enzyme : Trypsin_Strict (0) {R} {H} {Y}

Rank	(M+H)$^+$	delCn	Xcorr	SP	Ions	Reference	Protein
1	2335.1	0.0000	2.2358	253.7	17/38	owl | S68442 | S68442	human Gab1
2	2335.1	0.0174	2.1969	401.1	19/38	owl | S68442 | S68442	human Gab1

Peptides: 1) (R)HVSISYDIPPTPGNT#YQIPR
2) (R)HVSISYDIPPT#PGNTYQIPR

FIGURE 4: Identification of the ERK2-specific Gab1 phosphorylation site T312 by MALDI mass spectrometry. (A) MALDI mass spectra of fraction b46 (see Figure 2) were measured in linear (lin) mode as well as in reflector (ref) mode. Losses of phosphate marker ions ([M+H]$^+$-80 Da (loss of HPO_3^{2-}) and [M+H]$^+$-98 Da (loss of $H_3PO_4^-$)) were observed for the parent ion of *m*/*z* 2335.1 in the reflector mode. (B) Fragmentation mass spectrum of *m*/*z* 2335.1 parent ion. Designated b- and y-fragment ions allowed identifying a phosphorylated threonine residue (HVSISYDIPPT$_{P312}$PGNTYQIPR; aa 302−321) in the known primary structure of the peptide. Tp indicates the difference in fragment masses corresponding to the phosphorylated threonine residue. Beneath the spectrum, the result of computer-aided SEQUEST analysis is shown. The top-ranked choice by SEQUEST corresponds to the manually determined peptide sequence.

b46 revealed a phosphorylated peptide (HVSISYDIPPT-PGNTYQIPR) corresponding to the theoretical calculated peptide mass of Gab1 (amino acids 302−321) displayed in the single charged ion with *m*/*z* 2335.1 Da (Figure 3A). To elucidate the primary structure of all putative phosphopeptides the corresponding parent ion masses were subsequently selected for MALDI-PSD (post source decay) experiments. Figure 4B for example shows a MALDI-PSD spectrum of the Gab1 phosphopeptide HVSISYDIPPT$_{p312}$PGNTYQIPR identified in RP-HPLC fraction b46. The b- and y-ion series observed in the fragment mass spectra were used for automated interpretation by the Sequest algorithm (Figure

Table 1: Sequence Analysis Results of Gab1 Phosphopeptides

amino acid sequence of identified phosphopeptides[a]	$[M+H]^+b$	aa in Gab1[c]	phosphorylated residues	fraction[d]	part[e] of ^{32}P [%]
HVSISYDIPP**T**PGNTYQIPR	2335.1	302−321	T312	a/68, a/69, a/70, a/72, a/73, a/74, a/77, a/81, a/82, a/91, b/43, b/44, b/45, b/46, b/47, b/51, b/56, c/26	21
TASDTDSSYCIPTAGM**S**PSR	2197.8	365−384	S381	a/58, a/81, a/82, c/14, d/18	<1
NVLTVGSVSSEELDENYVPMNPN**S**PPRQHS	3212.5	431−460	S454	a/77, a/81, d/23	28
QHSSSFTEPIQEANYVPM**T**PGTFDFSSFGM QVPPPAHMGFR	4637.0	458−498	T476	a/91, a/92, a/94, b/61, b/62, c/36, c/37	17
VKPAPLEIKPLPEWEELQAPVR**S**PITR	3173.7	559−585	S581	a/77, a/81−82, b/51−52	15
FPM**S**PRPDSVHSTTSSSDSHDSEENYVPMN PNLSSEDPNLFGSNSLDGGSSPMIKPK	6186.7	594−650	S597	e/28−29, f/29−30	19

[a] Tryptic peptides of Gab1 were analyzed after anion exchange HPLC and reversed phase HPLC by mass spectrometry and Edman degradation. Phosphorylated S/T residues are marked in bold letters. Nontryptic digestion is underlined. [b] Single charged monoisotopic mass. [c] Numbering according to Gab1, Lehr et al. (*16*). [d] Fractions from anion exchange chromatography (letters)/reversed phase (numbers) HPLC (see Figure 3). [e] Total amount of radioactivity incorporated into the identified phosphopeptides corresponds to 100%.

4C) and allow the localization of phosphorylated amino acid residues in the primary structure of the phosphopeptide. In addition, theoretical values of b- and y-type ions for the determined phosphopeptides were calculated and compared to the fragment ions observed in the mass spectra to confirm the sequence assignment. Accordingly for the analyzed peptides locations of phosphorylated amino acid residues were determined and additionally validated by automated Edman degradation (data not shown).

Finally, we could associate two threonine and four serine residues (T_{312}, S_{381}, S_{454}, T_{476}, S_{581}, S_{597}) to the initially 32 analyzed RP-HPLC fractions (Table 1). Nearly the whole radioactivity incorporated (99%) in the identified phosphopeptides was equally distributed over five of the six identified phosphorylation sites being located in PXX(S/T)P or PX(S/T)P consensus motifs for MAP kinase phosphorylation (*26*). The remaining phosphorylation site (S_{381}) which claims less than 1% of the incorporated radioactivity is located in a truncated SP consensus motif for MAP kinase phosphorylation. Four of these sites (S454, S581, S597, T476) are located in the vicinity of three tyrosine residues (Y447, Y472, Y619), which appear to be binding sites for PI3K.

Functional Relevance of Identified ERK1/2 (p44/p42 MAPK) Specific Phosphorylation Sites. To dissect the functional relevance of ERK-specific Gab-1 phosphorylation in the context of insulin signaling in more detail, we investigated the ability of Gab1 to interact and activate PI3K in cellular context. Therefore, we generated a multiple Gab1 phosphorylation mutant (Gab1-ΔP) by changing serine S454, S581, S597 to alanine and threonine T476 to valine. Subsequently, CHO cells transiently transfected with Gab1 or Gab1-ΔP in the presence or absence of constitutive active MEK2 were used to perform PI3K-binding/activity assays from immunoprecipitated Gab1 protein complexes. Induction of CHO cells overexpressing Gab1 with insulin resulted in a substantial elevation of Gab1-associated PI3K binding/activity (Figure 5A,B) detected by Western blotting and PI3K activity assay. However, in the presence of ERK1/2 endogenous activated by MEK2 no significant change in PI3K binding/activity was detectable in response to insulin treatment. In contrast to that, using Gab1-ΔP, insulin stimulated PI3K activity was not significantly affected due to activated ERK1/2. To investigate a more downstream effect of ERK modulated insulin signal via Gab1 in intact cells, additionally we determined the activation level of Akt (Figure 5C). Treatment of CHO cells with insulin resulted in strong tyrosine phosphorylation of Gab1 and elevated levels of phospho-Akt. In contrast to that, in the presence of activated ERK1/2 levels of phospho-Akt were significantly reduced, without affecting tyrosine phosphorylation of Gab1.

DISCUSSION

The multifunctional docking protein Gab1 is activated and engaged in intracellular signaling of various RTKs, suggesting it plays an important role in the generation of biological responses for various cytokines and growth factors. A diversified set of biological responses are regulated by Gab1, e.g., mediating branching and alveolar morphogenesis of epithelial cells via HGF receptor activation and transformation of epithelial and NIH 3T3 cells (see for recent review refs *2* and *3*). RTK-mediated signal transduction via those multifunctional docking proteins is regulated by tyrosine and serine/threonine phosphorylation. Serine and threonine phosphorylation provides a regulatory mechanism at the level of docking proteins, i.e., allowing the modulation of the initial RTK signal.

Using a CHO cell system overexpressing the human insulin receptor, we could show that insulin-mediated activation of Akt via Gab1 was significantly reduced after endogenous activation of ERK. These results together with the observations made by Yu et al. (*18*, *19*) who demonstrated that ERK phosphorylation enhances HGF-dependent Gab1/PI3K but inhibits EGF-dependent Gab1/PI3K association and activation implicates that MAPK activation provides another specific regulatory mechanism which can result in divergent effects for distinct RTKs.

Therefore, to understand more about signal transduction via Gab1 we assigned the exact localization of the ERK 1/2-specific phosphorylation sites of Gab1. Because of the huge number (17) of potential S/T phosphorylation sites in classical consensus sequences for MAP kinases (S/T)P, PXX(S/T)P, and PX(S/T)P, we have chosen a protein chemistry approach combining HPLC and mass spectrometry as well as classic peptide sequencing approaches rather than the random in vitro mutation of all potential sites.

To guarantee monitoring of all successive separation steps enabling the selection of phosphopeptides containing frac-

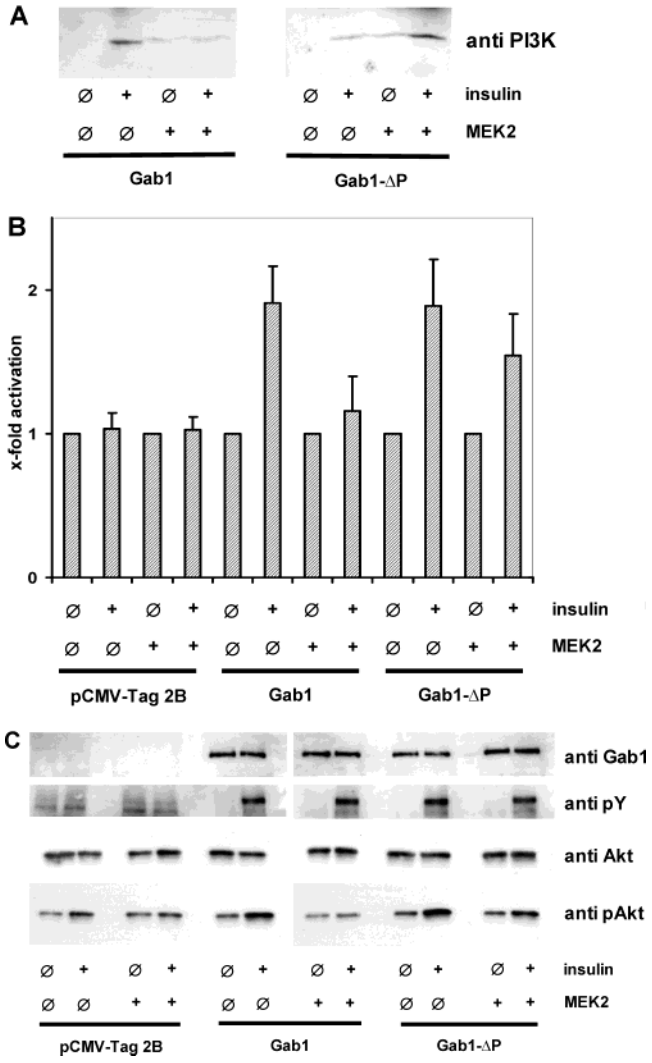


FIGURE 5: Role of ERK1/2 (p44/p42 MAPK) specific phosphorylation sites in Gab1 (S_{454}, T_{476}, S_{581}, and S_{597}) for insulin signal transduction. CHO cells overexpressing the human insulin receptor were transient-transfected with cloning vector (pCMV-Tag 2B), Gab1, Gab1-ΔP, and constitutive active MEK2 as indicated in the graphic. (A) PI3K-binding in response to treatment with insulin is shown. After induction of cells with insulin (1×10^{-7} M) for 3 min cells were harvested in lysis buffer as described in Experimental Procedures. Subsequently, aliquots of whole cell lysates (300 μg each) were used for immunopreciptiation of Gab1 protein complexes. Gab1 protein complexes were separated by SDS−PAGE and transferred to PVDF membranes (see Experimental Procedures). Blots were probed with anti-PI3K (p85 subunit; 1:1000) followed by chemiluminescence detection. A representative blot out of three independent experiments is displayed. (B) PI3K activity in response to treatment with insulin is shown. Activity of Gab1 associated PI3K (see panel A) was measured by ATP consumption in the phosphorylation reaction using Kinase-Glo Luminescent kinase assay (Promega) (see Experimental Procedures). Basal levels of kinase activity (without insulin) for each transfection couple are set to 1. Activity values of corresponding insulin treated samples are displayed as x-fold values. Results are given as mean ± SD of three independent experiments, each performed in duplicate ($p < 0.01$). (C) Activation of Akt in response to insulin is displayed. A total of 3 μg of whole cell lysates were separated by SDS−PAGE and transferred to PVDF membranes (see Experimental Procedures). Blots were initially probed with anti-phospho-Akt (1:1000) followed by chemiluminescence detection. To prove equivalent protein loading, blots were probed with anti-Akt (1:1000) as well as anti-Gab1 (1:1000) and with anti-pY (1:2000) to demonstrate phosphorylation of Gab1 on tyrosine residues, respectively. A representative blot out of three independent experiments is displayed.

tions as well as limiting the number of samples, in vitro phosphorylation of Gab1 was performed using radioactive tagged ATP. Subsequently, a two-dimensional HPLC approach utilizing anion exchange chromatography in combination with reversed-phase chromatography was applied to provide effective separation of hundreds of different peptides achieved by tryptic digestion of Gab1. Locations of the phosphorylated amino acid residues in the peptides then were analyzed by mass spectrometry. Phosphoserine and phosphothreonine containing peptides could be identified due to the loss of phosphate marker ions ($[M+H]^+$-80 Da (loss of HPO_3^{2-}) and $[M+H]^+$-98 Da (loss of $H_3PO_4^-$)) observed only in the reflector mode of a MALDI-MS instrument. Using MALDI-PSD-MS to receive information concerning the primary structure of the detected phosphopeptides, we identified two threonine and four serine residues that are phosphorylated by ERK2 (Table 1). To confirm MS results additionally classic peptide sequencing by Edman degradation was utilized.

In summary, using MALDI-PSD-MS and peptide sequencing we identified four serine and two threonine residues that are phosphorylated by ERK in vitro. Five of these phosphorylation sites (T_{312}, S_{454}, T_{476}, S_{581}, S_{597}) are corresponding to classic consensus motifs for MAP kinases (PXX(S/T)P or PX(S/T)P). One additionally serine residue (S_{381}) is located in a truncated consensus motif (SP). Interestingly, five of the six phosphorylation sites all located in complete consensus motifs representing more than 99% of overall incorporated radioactivity. Thereby phosphate incorporation is equally distributed over these sites. In contrast to that S_{381} exhibiting the truncated consensus motif was only marginally phosphorylated (less than 1%) suggesting it plays a negligible role in the cellular context.

Interestingly four (S_{454}, T_{476}, S_{581}, S_{597}) of the five preferentially phosphorylated S/T residues fitting nearly 80% of overall incorporated phosphate are located in the vicinity of tyrosine residues within YXXM motifs (Y_{447}, Y_{472}, Y_{619}), predicted to bind to the SH2 domain in the 85 kDa regulatory subunit (p85) of PI3K. Interestingly, these tyrosine residues represent the major phosphorylation sites specific for the IR and EGFR (*16*, *17*). Sequence alignment analyses of Gab1 demonstrate that the adjacent localization of YXXM and (S/T)P motifs were conserved within different species (human and mouse). Considering different related multifunctional docking proteins of the Gab and IRS family revealed that this specific constellation was also present in IRS1 and IRS2. In the case of Gab3 only one YXXM motif features such a configuration, whereas Gab2 and IRS4 do not fit to the described pattern.

To prove our observations in cellular context, we investigated the influence of ERK-specific phosphorylation on PI3K as well as its downstream effector Akt. Applied binding as well as activity assays pointed out that ERK phosphorylation of Gab1 is capable of blocking Gab1/PI3K-mediated insulin signal transduction, which resulted in reduced activation of Akt.

Taken together, these data suggest that the modulating effect of serine/threonine phosphorylation on Gab1/PI3K interaction is specific for the docking protein as well as the engaged RTK by which tyrosine phosphorylation is performed. In addition to site specific phosphorylation of docking proteins by different RTKs serine/threonine phos-

phorylation may provide another molecular mechanism for signaling selectivity and signal integration.

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