# Identification of Tyrosine Phosphorylation Sites in Human Gab-1 Protein by EGF Receptor Kinase in Vitro†

Stefan Lehr,‡ Jörg Kotzka,‡ Armin Herkner,‡ Elfriede Klein,‡ Christoph Siethoff,§ Birgit Knebel,‡ Volker Noelle,|| Jens C. Brüning,‡ Helmut W. Klein,|| Helmut E. Meyer,§ Wilhelm Krone,‡ and Dirk Müller-Wieland*,‡

*Klinik II und Poliklinik für Innere Medizin am Zentrum für Molekulare Medizin Köln and Institut für Biochemie der Universität zu Köln, Cologne, and Institut für Physiologische Chemie der Ruhr-Universität Bochum, Bochum, Germany*

*Received July 29, 1998; Revised Manuscript Received October 27, 1998*

ABSTRACT: Grb2-associated binder-1 (Gab-1) has been identified recently in a cDNA library of glioblastoma tumors and appears to play a central role in cellular growth response, transformation, and apoptosis. Structural and functional features indicate that Gab-1 is a multisubstrate docking protein downstream in the signaling pathways of different receptor tyrosine kinases, including the epidermal growth factor receptor (EGFR). Therefore, the aim of the study was to characterize the phosphorylation of recombinant human Gab-1 (hGab-1) protein by EGFR in vitro. Using the pGEX system to express the entire protein and different domains of hGab-1 as glutathione *S*-transferase proteins, kinetic data for phosphorylation of these proteins by wheat germ agglutinine-purified EGFR and the recombinant EGFR (rEGFR) receptor kinase domain were determined. Our data revealed similar affinities of hGab-1-C for both receptor preparations ($K_M = 2.7\ \mu$M for rEGFR vs 3.2 $\mu$M for WGA EGFR) as well as for the different recombinant hGab-1 domains. To identify the specific EGFR phosphorylation sites, hGab-1-C was sequenced by Edman degradation and mass spectrometry. The entire protein was phosphorylated by rEGFR at eight tyrosine residues (Y285, Y373, Y406, Y447, Y472, Y619, Y657, and Y689). Fifty percent of the identified radioactivity was incorporated in tyrosine Y657 as the predominant peak in HPLC analysis, a site exhibiting features of a potential Syp (PTP1D) binding site. Accordingly, GST-pull down assays with A431 and HepG2 cell lysates showed that phosphorylated intact hGab-1 was able to bind Syp. This binding appears to be specific, because it was abolished by changing the Y657 of hGab-1 to F657. These results demonstrate that hGab-1 is a high-affinity substrate for the EGFR and the major tyrosine phosphorylation site Y657 in the C terminus is a specific binding site for the tyrosine phosphatase Syp.

Signal transduction by receptor tyrosine kinases (RTK) involves phosphorylation of different intracellular signaling proteins and their interaction via, for example, specific phosphotyrosine-recognizing protein domains, like SH2 domain proteins. Therefore, intracellular signaling appears to be determined by a complex network of protein−protein interactions regulated by tyrosine phosphorylation. Recently, a novel member of a multisubstrate docking protein has been characterized by screening an expression cDNA library from a glial tumor using recombinant Grb2, called Gab-1 (Grb2-associated binder-1) (*1*). Gab-1 is expressed in many tissues, and there is some evidence that it is a part of signaling pathways related to cell growth and transformation as well as apoptosis (*2*−*5*).

Structural and functional features of Gab-1 indicate that it belongs to the family of insulin receptor substrate (IRS) proteins (for a recent review, see ref *6*). Gab-1 contains an N-terminal PH domain, two SH3-binding proline-rich regions, and several potential tyrosine phosphorylation sites likely to represent SH2-binding motifs. The PH domain of Gab-1 appears to be functionally homologous to other IRS proteins, because in contrast to PH domains from non-IRS-like signaling molecules the PH domain of Gab-1 can couple as a chimeric protein IRS-1 phosphorylation to the insulin receptor (*7*). Phosphorylated Gab-1 appears to recruit downstream signaling elements, like the 85 kDa regulatory subunit (p85) of the PI-3 kinase, phospholipase C$\gamma$ (PLC-$\gamma$), and the protein tyrosine phosphatase 1D (PTP1D; Syp) (*1*).

Gab-1 is phosphorylated by stimulation with insulin and several other growth factors, like the nerve growth factor (NGF) (*4*), basic fibroblast (FGF) (*2*), hepatocyte growth factor (HGF) (*8*), and epidermal growth factor (EGF) (*1*).

Therefore, as a multifunctional adapter protein, Gab-1 can converge as a result different extracellular stimuli and recruit various intracellular signaling proteins. Distinct phosphorylation patterns of Gab-1 evoked by interaction with different activated receptor kinases might be the molecular basis for

† This study was supported by the German Research Foundation (DFG/SFB-351 and DFG/ME 765/3-4), Fritz Thyssen Stiftung, the German Ministry of Education and Research (BMBF/01 KS 9502), and the Graduierten Kolleg (Molekulare Mechanismen pathophysiologischer Vorgänge, DFG) to S.L.

* To whom correspondence should be addressed: Klinik II und Poliklinik für Innere Medizin der Universität zu Köln, 50924 Cologne, Germany. Telephone: 49-221-478-4070. Fax: 49-221-478-4179.

‡ Klinik II und Poliklinik für Innere Medizin am Zentrum für Molekulare Medizin Köln der Universität zu Köln.

§ Institut für Physiologische Chemie der Ruhr-Universität Bochum.

|| Institut für Biochemie der Universität zu Köln.

10.1021/bi9818265 CCC: $18.00 
Published on Web 12/08/1998

signaling selectivity. In this report, we identify the tyrosine phosphorylation sites in human Gab-1 (hGab-1) related to the EGF receptor kinase in vitro.

## EXPERIMENTAL PROCEDURES

*Construction and Expression of Human Recombinant GST Fusion Proteins*. cDNA from human skeletal muscle (Quick-clone, Clontech) was used to amplify the whole coding sequence (nucleotides 122−2206, according to ref *1*) from the Grb2-associated binder-1 (Gab-1) gene by polymerase chain reaction using Expand polymerase (Boehringer Mannheim) and oligonucleotide primers, including appropriate restriction endonuclease consensus sequences to allow cloning into the *Eco*RI (underlined sequence) site of the pGEX-3X expression vector (5′-primer GGAATTCAATGAGCGGTGGTGAAGTGGT, 3′-primer GGAATTCCCTCATTTCACACTCTTCGGTGGCGT). Three fragments were amplified using oligonucleotide primers, including appropriate restriction endonuclease consensus sequences to allow asymmetric cloning into the *Bam*HI−*Eco*RI (underlined sequence) sites of the pGEX-3X expression vector (nucleotides 122−499, 5′-primer GGGGATCCCCATGAGCGGTGGTGAAGTGGT, 3′-primer GGAATTCTTCACAGGATCTT; nucleotides 788−1696, 5′-primer GGGGATCCCTGCTTCCTCCCAGA, 3′-primer GGAATTCGGCTTGAGGTTCCTA; and nucleotides 1826−2206, 5′-primer GGGGATCCGACCAGATTCAGTGCA, 3′-primer GGAATTCCCTCATTTCACACTCTTCGGTGGCGT). Accordingly, four human Gab-1−GST fusion proteins were purified from transformed bacteria (BL21) according to the manufacturer's recommendations (Pharmacia), i.e., entire GST fusion protein (C), amino acids (aa) 1−724 with an apparent $M_r$ of 105.6; N-terminal (NT), aa 1−126 with an apparent $M_r$ of 39.9; middle domain (MD), aa 223−525 with an apparent $M_r$ of 59.2; and C-terminal (CT), aa 599−724 with an apparent $M_r$ of 39.8. Generation of a complete human genomic Gab-1 clone using recombinant DNA techniques and screening of a human genomic library (Stratagene 946206) with $^{32}$P-labeled DNA probe (cDNA; nucleotides 122−777) were performed according to standard procedures (*9*). The identity of the human genomic Gab-1 clone was verified by restriction endonuclease analyses and nucleotide sequencing (*10*).

*EGF Receptor (EGFR) Kinase Preparation*. Human hepatoma cells (HepG2) and epidermal cells (A431) were cultured at 37 °C in 5% $CO_2$ (v/v) and RPMI 1640 medium supplemented with 10% fetal bovine serum and antibiotics (Life Technologies, Inc.). For receptor preparation, HepG2 as well as A431 cells were incubated for 24 h in serum-free medium before receptor purification by wheat germ agglutinine (WGA) (*11*). The recombinant EGF receptor (rEGFR) containing the human EGFR amino acid sequence of residues 649−1186 (*12*) was expressed and purified by slightly modifying (H. W. Klein, personal communication) a baculovirus expression system as described previously (*13*).

*Substrate Phosphorylation Assay*. For GST fusion protein phosphorylation (*14*) by WGA-purified receptors, the WGA column-eluted protein was preincubated for 60 min at 4 °C with 10 nM EGF in 50 mM HEPES/NaOH (pH 7.5), 5 mM $MgCl_2$, 5 mM $MnCl_2$, 1 mM DTT, 1 mM $Na_3VO_4$, 1 mg/mL BSA, and 0.1% Triton X-100. Autophosphorylation was initiated by addition of ATP and [$\gamma$-$^{32}$P]ATP to final concentrations of 250 $\mu$M and 0.1 mCi/mL, respectively, and continued during incubation for 10 min at 22 °C. Substrate phosphorylation was initiated by addition of autophosphorylated receptor (equivalent to 2.5 $\mu$g of WGA-eluted protein) to aliquots of the different GST fusion proteins in kinase buffer [50 mM HEPES/NaOH (pH 7.5), 5 mM $MgCl_2$, 5 mM $MnCl_2$, 1 mM DTT, 1 mM $Na_3VO_4$, 250 $\mu$M ATP, 0.1 mCi/mL [$\gamma$-$^{32}$P]ATP, and 1 mg/mL BSA] to achieve substrate concentrations of 0.25−128 $\mu$M. The reaction was allowed to proceed for 180 s at 22 °C and terminated by addition of 2× sample buffer [50 mM Tris-HCl (pH 6.8), 100 mM DTT, 10% glycerol, 2% SDS, and 0.1% bromphenol blue] followed by boiling for 5 min. Phosphorylated proteins were separated by 10% SDS−PAGE (*15*) and analyzed by autoradiography of the Coomassie-stained and dried gels. Extents of phosphate incorporation were determined by Cerenkov counting of excised fragments.

To phosphorylate substrate with recombinant receptor kinase domains, rEGFR was autophosphorylated for 5 min at 22 °C in kinase buffer with 1 $\mu$M poly(lysine). Aliquots of autophosphorylated recombinant receptors (2.5 pmol) were used to perform substrate phosphorylation for 60 s at 22 °C. Substrate concentrations ranged from 0.25 to 32 $\mu$M. Termination of the reaction and analysis of phosphate incorporations were performed as described above. Conversion of protein concentration into molar units was based on the calculated molecular mass of the GST fusion proteins. The $K_M$ values were determined using the Enzfitter program (Elsevier, Biosoft). For phosphoamino acid analysis of the fusion proteins, aliquots of different proteins at a substrate concentration of 30 $\mu$M were incubated with autophosphorylated EGFR and rEGFR under the conditions described above. Phosphoamino acid analysis was performed essentially as described by Kamps (*16*).

*High-Performance Liquid Chromatography (HPLC) and Nanospray Mass Spectrometry*. Using 0.3 nmol of recombinant EGFR, 5 nmol of Gab-1-C protein was phosphorylated with [$\gamma$-$^{32}$P]ATP for 25 min under the conditions described above. The proteins were separated by SDS−PAGE, and phosphorylated hGab-1-C was digested with 50 $\mu$g of trypsin (sequencing grade, Boehringer Mannheim) in the excised gel pieces overnight at 37 °C (*17*, *18*). The peptides were eluted with 50 mM ammonium carbonate and separated on an anion exchange column (Nucleogel SAX 1000-8/46, 50 mm × 4.6 mm, Machery & Nagel) using the Beckman gold solvent delivery system. The HPLC flow rate was 0.5 mL/min. After injection of 1 mL of the sample, the peptides were eluted beginning at 100% buffer A [20 mM $NH_4CH_2COOH$ (pH 7.0)] and 0% buffer B [0.5 M $KH_2PO_4$ (pH 4.0)]. The amount of buffer B was increased to 10% over the course of 40 min and from 10 to 50% over the course of 75 min. Fractions of 0.5 mL were collected. Radioactive fractions were subjected to reversed phase HPLC. The peptides were separated on a C18 reversed phase column (150 mm × 0.8 mm, 5 $\mu$M particle size, 300 Å pore size, LC Packings, The Netherlands) using the ABI 140 B solvent delivery system (Applied Biosystems). The HPLC flow rate was adjusted to 70 $\mu$L/min via a titanium t-piece. After application of 100 $\mu$L of the sample, elution started with 95% solution A (0.1% TFA) and 5% solution B [acetonitrile/water/TFA (84/16/0.1, v/v/v)]. The content of solution B was raised to 50% over the course of 90 min and from 50 to 100% over the course of

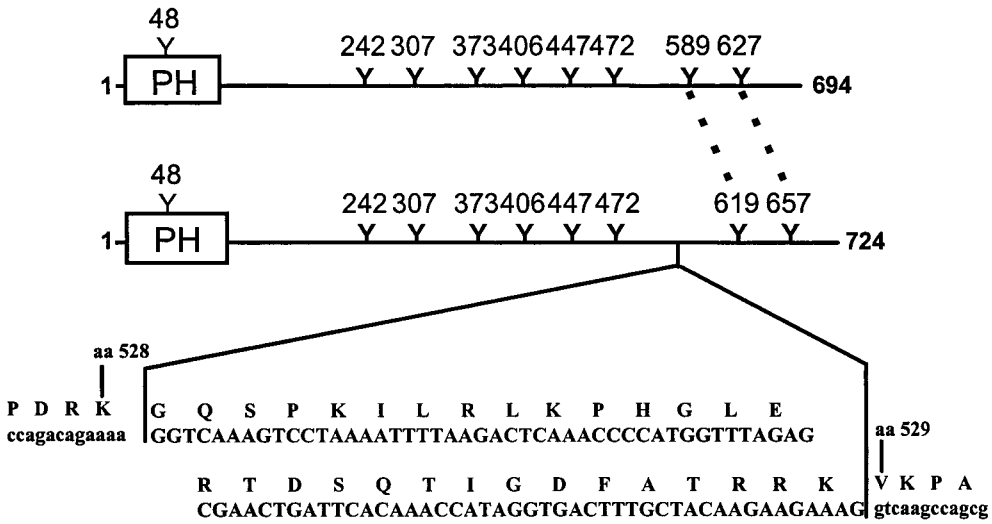


FIGURE 1: Structure of human Gab-1. cDNA from human skeletal muscle was used to amplify the whole coding sequence of Gab-1 previously published (*1*), encoding a protein of 694 amino acids containing a pleckstrin homology (PH) domain as shown graphically. The sequence of our cDNA clone contained in frame 90 additional nucleotides encoding 30 amino acids (aa) between aa 528 and 529 and a conservative exchange of one nucleotide (indicated with a capital A, codon of aa 527). The upper part represents the change in structure; i.e., Gab-1 consists of 724 aa instead of 694. Potential tyrosine phosphorylation sites predicted to bind SH2 domains change from (Y) 589 and 627 to 619 and 657, respectively.

15 min. The collected fractions containing radiolabeled peptides were subjected to Edman degradation with an Applied Biosystems model 476A pulse-liquid sequenator (*19*) and mass spectrometry, which was performed on a TSQ 7000 instrument (Finnigan) equipped with a nanoelectrospray ion source. The mass spectra were aquired in the positive ion mode in the mass range of *m*/*z* 40−2500 with a scan duration of 2 s. The electrospray voltage was held to approximately 1 kV. Collision-activated dissociation spectra were obtained using argon as the collision gas with a pressure of approximately $4 \times 10^{-3}$ mbar. The collision energy was set between 15 and 46 eV depending on the parent ion *m*/*z* ratio. In addition to manual comparison of the known sequence, the SEQUEST program (OWL sequence database, University of Leeds, Leeds, U.K.) was also used to identify the peptide phosphorylation sites (*20*, *21*).

*Cellular Fractions*. HepG2 and A431 cells were cultured as described above. After incubation for 24 h with serum-starved medium, the cells were washed twice with PBS. Cells were lysed in pull down (PD) buffer [50 mM Hepes (pH 7.5), 1% (v/v) Nonidet P-40, 150 mM NaCl, 5 mM EDTA, 10 mM NaF, 1 mM $Na_3VO_4$, and 1 mM glycerophosphate supplemented with Complete (Boehringer Mannheim)]. Insoluble material cell lysates were removed after centrifugation at 12000*g* for 10 min at 4 °C.

*GST Pull Down Assay*. In vitro phosphorylated hGab-1 proteins (5 $\mu$g), nonphosphorylated hGab-1 proteins (5 $\mu$g), and GST (5 $\mu$g) were incubated with proteins of A431 and HepG2 lysates (each 1 mg) for 30 min at 4 °C. Glutathion sepharose beads [50 $\mu$L of a 50% (v/v) slurry, Pharmacia] were added and samples incubated at 4 °C on a rotator (60 rpm) for 1 h. Pellets were washed three times with PD buffer. Bound proteins were eluted with 40 $\mu$L of 1× SDS−PAGE sample buffer and separated by SDS−PAGE.

*SDS−PAGE and Western Blotting*. SDS−PAGE was performed according to Laemmli (*15*) followed by electroblotting (*22*) to PVDF membranes (Bio-Rad) for probing with antibodies detected by BM chemiluminescence (Boehringer Mannheim). The following primary antibodies were used at concentrations recommended by the suppliers: anti-phosphotyrosine monoclonal (RC20), anti-Syp monoclonal (Transduction Laboratories), and anti-Gab-1 monoclonal (Upstate Biotechnology). Kodak Xomat AR film was used for detection of chemiluminescence signals. Before reprobing, blots were incubated with stripping solution (100 mM 2-mercaptoethanol, 2% SDS, and TBS) at 55 °C for 60 min. The washed blots were reblocked prior to reprobing.

*Site-Directed Mutagenesis*. The Tyr 48 → Phe 48 and Tyr 657 → Phe 657 mutants of hGab-1-C were made by site-directed mutagenesis using the Quick Change Kit (Stratagene) according to the manufacturer's recommendations. The following primers were used: Tyr 48 → Phe 48, 5′-CTGGAGATCCAGATGTTTTGGAATATTT**T**CAAAAAT-GATCATGCC; and Tyr 657 → Phe 657, 5′-GGAGAC-AAACAGGTGGAAT**T**CTTAGATCTCGAC.

## RESULTS

To construct recombinant human Grb2-associated binder-1 (Gab-1) proteins, we used cDNA from human skeletal muscle to amplify the whole coding sequence from hGab-1 [previously published (*1*)]. It is interesting to note that the sequence of our cDNA clone contained in frame 90 additional nucleotides encoding 30 amino acids between aa 528 and 529 of the published sequence (see Figure 1). This sequence does not contain a potential phosphotyrosine residue and was verified by isolating and sequencing clones of a human genomic library (data not shown). Since all our cDNA and genomic clones contained the additional nucleotide sequence, it is highly unlikely that our sequence represents a simple splice variant.

To express the entire protein and different domains of hGab-1 as glutathione *S*-transferase proteins (Figure 2A), the pGEX system was used. Purification of the recombinant proteins from the soluble fraction of bacterial extracts was achieved by affinity chromatography using glutathione

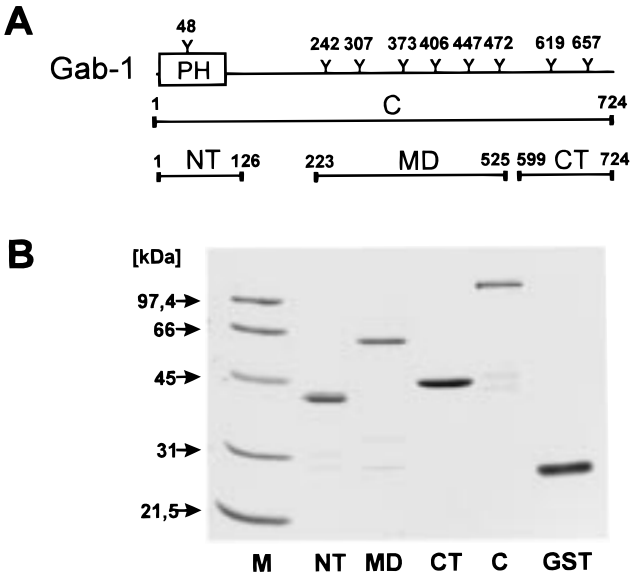


FIGURE 2: Structure, nomenclature, and expression of human Gab-1 fusion proteins. (A) Structure and nomenclature of hGab-1 proteins. The pleckstrin homology domain is abbreviated as PH. Predicted binding sites of SH2 domains representing potential phosphotyrosines are indicated with a Y. The pGEX system was used to express the entire protein (C, aa 1−724) and different domains of hGab-1 as glutathione *S*-transferase proteins, i.e., N-terminal sequence (NT, aa 1−126), middle domain (MD, aa 223−525), and C-terminal region (CT, aa 599−724). (B) SDS−PAGE analysis of hGab-1 fusion proteins. Aliquots from purified hGab-1 proteins (2 $\mu$g each) using affinity chromatography on glutathione sepharose were analyzed by Coomassie blue staining after 10% SDS−PAGE. Lane M contained the prestained low-range marker; GST is glutathione *S*-transferase.

Table 1: $K_M$ Values for hGab-1 Phosphorylation by EGF Receptors[a]

| receptor domain | EGFR | rEGFR |
|---|---|---|
| hGab-1-NT | 23.7 ± 4.4 $\mu$M | 4.0 ± 0.4 $\mu$M |
| hGab-1-MD | 14.9 ± 2.0 $\mu$M | 4.9 ± 1.5 $\mu$M |
| hGab-1-CT | 10.0 ± 2.4 $\mu$M | 2.4 ± 0.5 $\mu$M |
| hGab-1-C | 3.2 ± 0.4 $\mu$M | 2.7 ± 0.5 $\mu$M |

[a] Phosphorylation of entire hGab-1 and domains (see Experimental Procedures) catalyzed by EGFR. Values obtained using partially purified EGF (EGFR) preparations and recombinant EGF (rEGFR, aa 649−1186) are shown. Mean values ± SD of each of four independent experiments are given. Methods used for phosphorylation reactions and determination of $K_M$ values are described in Experimental Procedures. The corresponding kinetics are shown in Figure 3.

Sepharose with an overall yield of 2 mg/L of bacterial culture. Estimation by Coomassie-stained gels showed a purity of all hGab-1 fusion proteins exceeding 90% (Figure 2B). Kinetic data for the entire hGab-1 (hGab-1-C) and different recombinant hGab-1 domains (Figure 2A) phosphorylated by recombinant and partially purified EGF receptor are displayed in Figure 3. Using hGab-1-C as the substrate, our data revealed a similar affinity for both receptor preparations ($K_M$ = 2.7 $\mu$M for rEGFR vs 3.2 $\mu$M for WGA EGFR) as well as for the different recombinant hGab-1 domains, which are summarized in Table 1. However, apparently affinities of either receptor preparation were highest for the entire substrate protein.

Phosphoamino acid analyses revealed phosphorylation of hGab-1 proteins by recombinant and WGA-purified native EGFR exclusively on tyrosine residues under our experimental conditions (data not shown). Upon trypsinizing hGab-1-C, we identified the EGFR specific tyrosine phosphorylation sites by separating the peptides from rEGFR-phosphorylated [$^{32}$P]hGab-1-C (5 nmol) by anion exchange HPLC (*23*). The phosphorylated [$^{32}$P]hGab-1-C was digested in gel with trypsin, and the eluted peptides were separated by anion exchange HPLC. More than 90% of the radioactivity was recovered from the gel after digestion, and between 80 and 90% of the radioactive peptides were eluted from the column. hGab-1-C phosphorylation with both receptors revealed identical phosphopeptide patterns resulting from anion exchange HPLC. The radioactivity profile analyzed by anion exchange HPLC of phosphorylated hGab-1-C showes equal phosphorylation patterns independent of phosphorylation time (data not shown). The radioactivity (Cerenkov) elution profile of anion exchange HPLC showed nine major peaks (a−i), with a predominant peak occurring with anion exchange HPLC fraction 67 (Figure 4A). The radioactive fractions of each peak were pooled according to the elution profile and subjected to reversed phase HPLC (Figure 4a−i). We identified 17 different radiolabeled fractions. These radiolabeled peptides were subjected to automated Edman degradation and electrospray ionization (ESI) mass spectrometry. In Table 2, we summarize the identified phosphorylation sites of the phosphopeptides derived from the complete protein. We identified eight EGFR specific tyrosine phosphorylation sites in hGab-1-C. Six of the identified phosphorylation sites (Y373, Y406, Y447, Y472, Y619, and Y657) were identical to the postulated sites (*1*). For the EGFR, there was no detectable phosphorylation evidence of the three postulated N-terminal tyrosine phosphorylation sites (Y48, Y242, and Y307) in the hGab-1-C, but we recovered two phosphotyrosine-containing peptides in other hydrophobic motifs (Figure 5). The predominant tyrosine phosphorylation site (Y657) in the peptide corresponding to the amino acid residues 654−666 (QVEY*LDLDLDSGK) was observed. Nearly 50% of the radioactivity incorporated in the identified phosphopeptides was present in this phosphopeptide. It also corresponded to the major peak in anion exchange HPLC. Tyrosine 657 exhibited features of a potential PTP1D (Syp) binding site (*1*). In addition, 20% of the radioactivity was present in a peptide corresponding to amino acid residues 687−696 (VDY*VVVDQQK). This peptide exhibited no potential binding motive for known SH2 domain proteins. The other identified phosphorylated peptides occupied 0.7−15% of the radioactivity found in the identified peptides.

The functional relevance of these data was tested by GST pull down assays with whole lysates of A431 and HepG2 cells. First, we investigated the ability of phosphorylated hGab-1-C in comparison to that of unphosphorylated protein and GST to bind Grb2 in vitro. Phosphorylated hGab-1-C showed a higher affinity for Grb2 from A431 and HepG2 cell lysates than unphosphorylated protein, regardless of the extent of phosphorylation by native EGFR or rEGFR (Figure 6A). With GST as a control, no binding of Grb2 could be detected. To prove that equivalent concentrations of hGab-1-C are loaded in all lanes, the blots were stripped and reprobed with anti-Gab-1 antibodies. To demonstrate tyrosine phosphorylation, the blots were stripped and reprobed with anti-phosphotyrosine antibodies. Exclusively in the lanes with native or recombinant EGFR, hGab-1 phosphorylation on tyrosine residues occurred. Y48 was postulated to display a

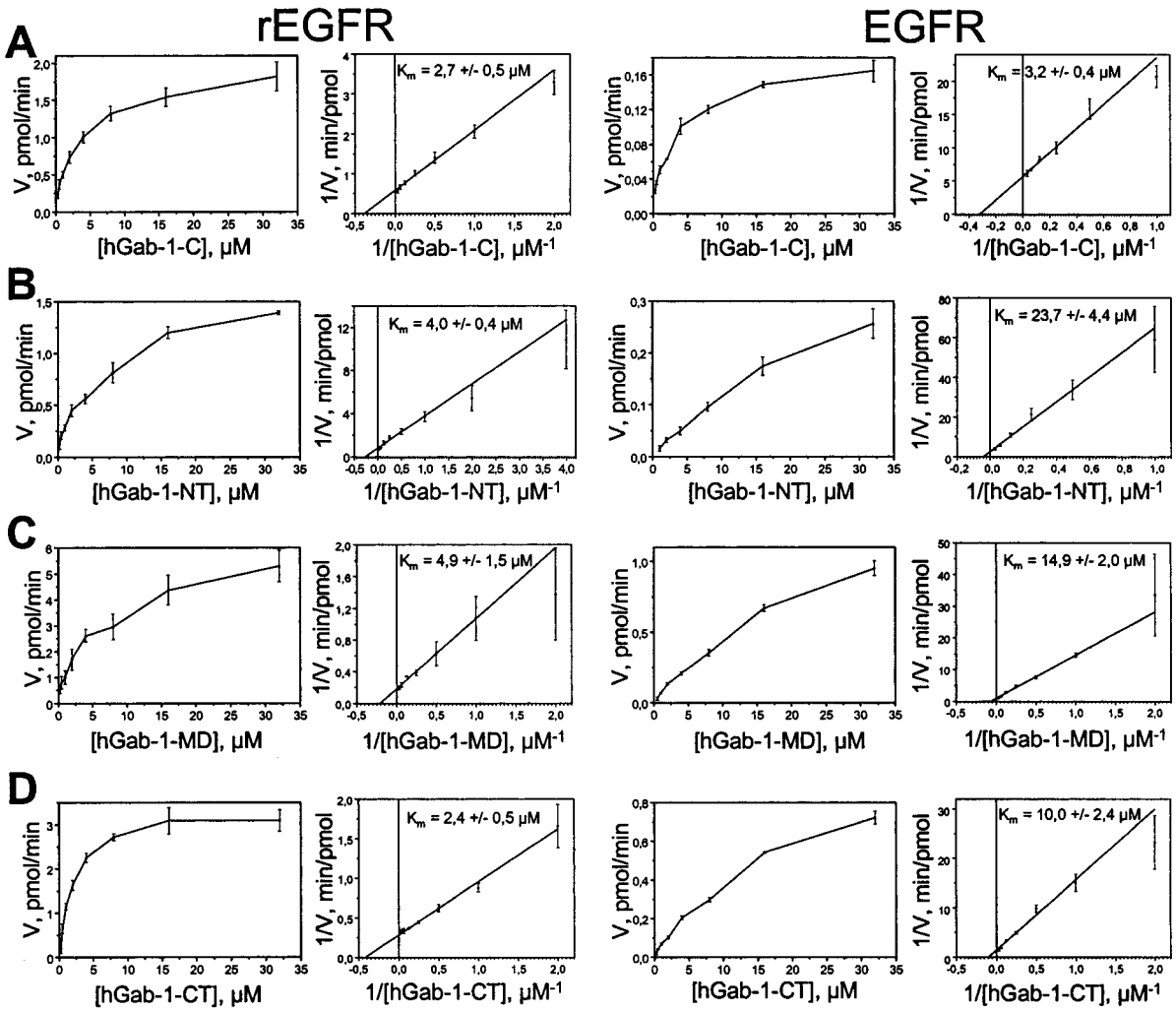


FIGURE 3: Michaelis−Menten kinetics of hGab-1 with the EGF receptor. The extent of phosphorylation of hGab-1 catalyzed by WGA-purified (EGFR) or recombinant EGF receptor (rEGFR, aa 649−1186) is shown using (A) the entire hGab-1 protein (C, aa 1−724) or different domains of hGab-1 as glutathione *S*-transferase proteins, i.e., (B) N-terminal sequence (NT, aa 1−126), (C) middle domain (MD, aa 223−525), and (D) C-terminal region (CT, aa 599−724). Velocity ($v$) vs substrate concentration and double-reciprocal plots are shown. Mean values ± standard deviation (SD) of four independent experiments were depicted in the plots. Methods used for phosphorylation reactions and determination of $K_M$ values are described in Experimental Procedures.

Table 2: Sequence Analysis Results of hGab-1 Phosphopeptides[a]

| amino acid sequence of the identified phosphopeptide | aa in hGab-1[b] | phosphorylated residue | fraction[c] | part[d] of $^{32}$P (%) |
|---|---|---|---|---|
| VSPSSTEADGEL**Y**VFNTPSGTSSVETQMR | 273−301 | Y285 | c32 and e10/13 | 14.9 |
| TASDTDSS**Y**CIPTAGMSPSR | 365−384 | Y373 | c20 | 5.2 |
| DASSQDC**Y**DIPR | 399−410 | Y406 | f6 | 1.6 |
| NVLTVGSVSSEELDEN**Y**VPMNPNSPPR | 431−457 | Y447 | f15 | 8.8 |
| QHSSSFTEPIQEAN**Y**VPMTPGTFDFSSFGMQVPPPAHMGFR | 458−498 | Y472 | b37 | 2.5 |
| PDSVHSTTSSSDSHDSEEN**Y**VPMNPNLSSEDPNLFGSNSLDGGSSPMIK | 600−648 | Y619 | g20 | 0.7 |
| QVE**Y**LDLDLDSGK | 654−666 | Y657 | h8/9 and i14 | 47.4 |
| VD**Y**VVVDQQK | 687−696 | Y689 | c15 and d13 | 19.7 |

[a] Tryptic peptides of hGab-1-C were analyzed after anion exchange HPLC and reversed phase HPLC by mass spectrometry and Edman degradation. [b] Numbering according to Holgado-Madruga et al. (*1*) modified by DNA sequence analysis (see also Figure 1). [c] Fractions from anion exchange chromatography (letters)/reversed phase (numbers) HPLC (see Figure 4). [d] Incorporated radioactivity of the identified phosphopeptides is set to 100%.

Grb2 binding site, but no phosphorylation of this site could be detected. To test the relevance of tyrosine Y48 in Grb2 interaction, Y48 was altered by site-directed mutagenesis to F48. The mutant did not exhibit a different Grb2 binding pattern in comparison to the wild type protein.

Y657, which shows features of a Syp binding site, represents the main EGFR phosphorylation site of hGab-1-C. We investigated the ability of hGab-1-C to bind Syp in vitro. Phosphorylated hGab-1-C showed a high affinity for binding Syp, whereas unphosphorylated hGab-1-C failed to bind Syp (Figure 6B). The ability of phosphorylated Y657 to bind Syp was tested by changing Y657 to F657. Upon this exchange, no binding of Syp could be observed using phosphorylated hGab-1-$C_{Y657F}$. Additionally, the binding

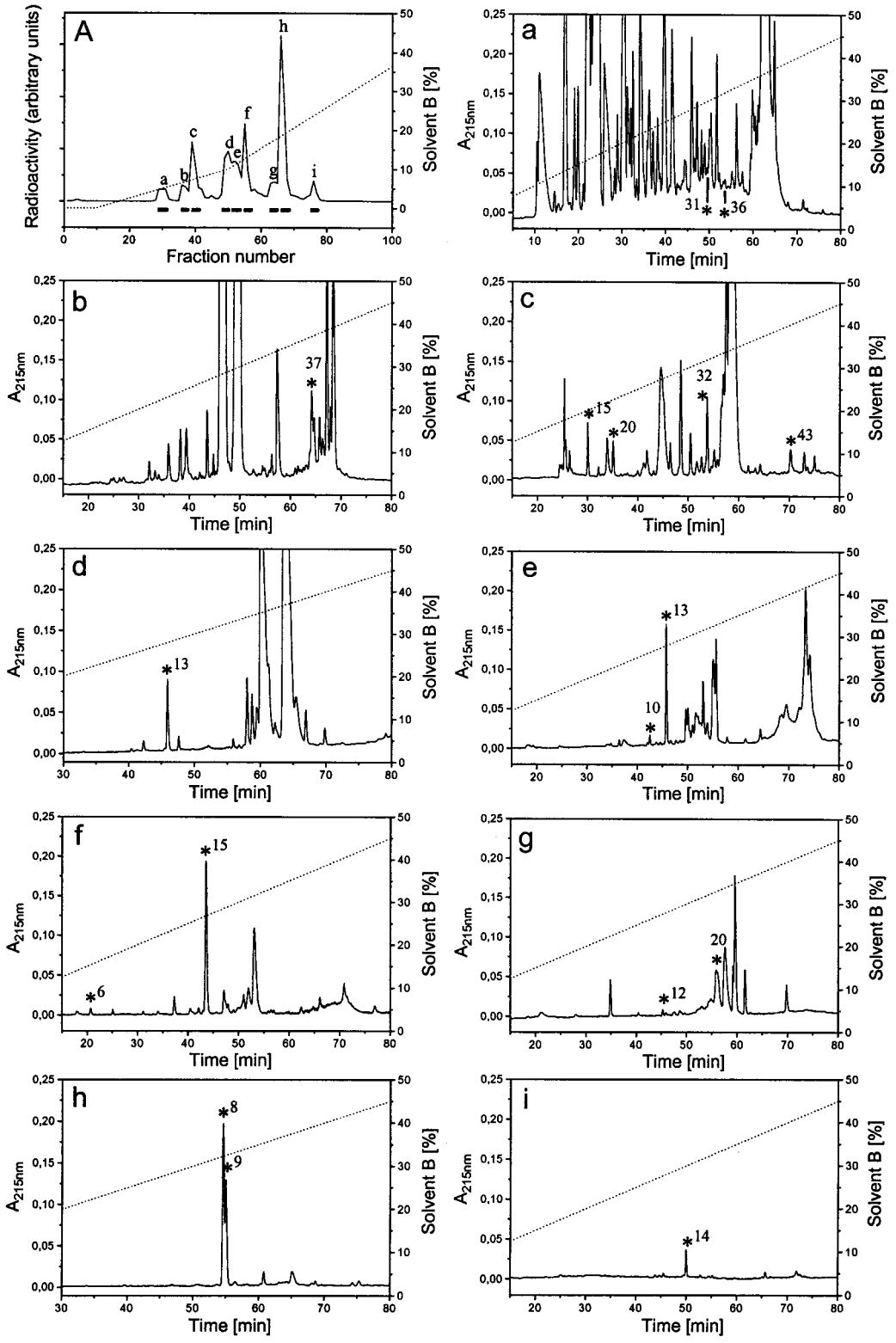


FIGURE 4: HPLC analysis of tryptic phosphopeptides derived from in vitro-phosphorylated hGab-1. Five nanomoles of hGab-1-C was phosphorylated for 25 min with 0.3 nmol of rEGFR. The phosphorylation reaction was performed as described in Experimental Procedures. Proteins were separated by SDS−PAGE, and the excised hGab-1 was digested with trypsin. (A) HPLC anion exchange chromatography of the tryptic digest. Radioactive fractions were pooled according to the solid bars. Pooled fractions a−i were subjected for rechromatography to C18 reversed phase HPLC. (a−i) Reversed phase HPLC elution profiles of anion exchange HPLC fractions a−i are shown. Radioactive fractions are designated with asterisks followed by the fraction number.

pattern of Grb2 showed no significant difference between hGab-1-C and $hGab_{Y657F}$.

Pull down assays with hGab-1-CT (Figure 6C) also displayed a phosphorylation-dependent interaction with Syp. Binding of Grb2 could not be detected regardless of the extent of hGab-1-CT phosphorylation, which contains the two major tyrosine phosphorylation sites but no SH3 domain binding sites. In contrast to that, we observed a constitutive, phosphorylation-independent association of Grb2 with the middle portion of hGab-1 (data not shown), which contains

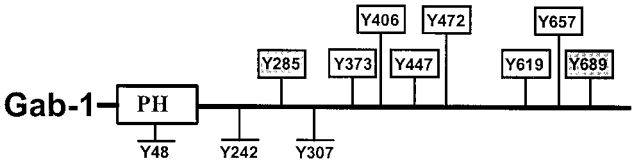


FIGURE 5: Potential tyrosine phosphorylation sites of hGab-1-C. Phosphorylation sites (for EGFR) identified (Edman sequencing and ESI mass spectrometry) in this report are listed above the line. Tyrosines which correspond to postulated phosphorylation sites are shown as white boxes, whereas sites without known binding motifs for SH2 domain proteins are shown as gray boxes. Other postulated phosphorylation sites listed below the line are not present in a phosphorylated state.

two SH3 domain binding sites.

## DISCUSSION

Insulin receptor substrates IRS-1 (*24*), IRS-2 (*25*), IRS-3 (*26*), IRS-4 (*27*), and the recently identified Grb-2 binding protein Gab-1 share some structural and functional features, indicating that they are members of an intracellular multifunctional "docking" protein family (*6*, *7*, *24*−*27*). It has been shown that IRS-1 and IRS-2 are phosphorylated not only by members of the insulin receptor family but also by treating cells with other hormones and cytokines, but not with EGF or PDGF (*28*). In contrast to this, Gab-1 appears to be phosphorylated by stimulation of cells with EGF (*1*). It can also associate with the c-Met receptor tyrosine kinase (*29*) and might recruit different intracellular signaling proteins. It has been shown, for example, that IRS-1 and IRS-2 do not bind phospholipase $C_\gamma$ (*30*), whereas Gab-1 does. Differential extents of phosphorylation by different stimuli of these intracellular docking proteins might be a mechanism by which the same protein can activate specific downstream pathways. In this study, we show tyrosine phosphorylation kinetics of hGab-1 in vitro, indicating that hGab-1 is a high-affinity substrate of the EGF receptor. Furthermore, we identified the main EGFR specific tyrosine phosphorylation sites of hGab-1-C by Edman degradation and mass spectrometry.

*Gab-1, a High-Affinity Substrate of the EGF Receptor*. Phosphorylation of the hGab-1-C fusion protein by rEGFR or partially purified native EGF receptor produces no significant difference in $K_M$ values. $K_M$ values of different recombinant hGab-1 proteins determined by the native EGFR indicated a rank order of affinity: NT < MD < CT < C. This might be evidence of a more complex substrate−receptor interaction, which is provided indirectly by the fact that the required substrate concentration for half-maximal saturation ($K_M$) is lower for the intact hGab-1 than for separate hGab-1 domains. A similar phenomenon has been observed for IR-stimulated phosphorylation of IRS-1 in vitro (*14*). Values after substrate phosphorylation of a human recombinant IRS-1 domain (aa 586−1149, called hIRS-p30) by WGA-purified IR preparations are in the range of 6−12 $\mu$M, whereas kinetic constants determined by the use of peptides with YXXM and YMXM motifs of IRS-1 are in the range of 24−300 $\mu$M (*31*). There is almost no difference in $K_M$ values between WGA-purified or recombinant receptor preparations when hIRS-p30 is used as the substrate (*14*). The $K_M$ values obtained for hGab-1 and the EGF receptor are similar. This shows that IRS-1 appears to be a high-affinity substrate for the insulin receptor and hGab-1 for the EGF receptor.

*Identification of EGFR Specific Tyrosine Phosphorylation Sites in hGab-1*. Gab-1 contains at least 17 potential tyrosine phosphorylation sites, including nine binding sites for known SH2 domain motifs enabling its interaction with PI-3 kinase, Grb2, phospholipase $C_\gamma$, and Syp (*1*). We separated the tryptic peptides of EGFR-phosphorylated hGab-1-C by a combination of two different successive HPLC protocols. Using peptide sequencing and ESI mass spectrometry, we identified the phosphorylated tyrosine residues of the result-

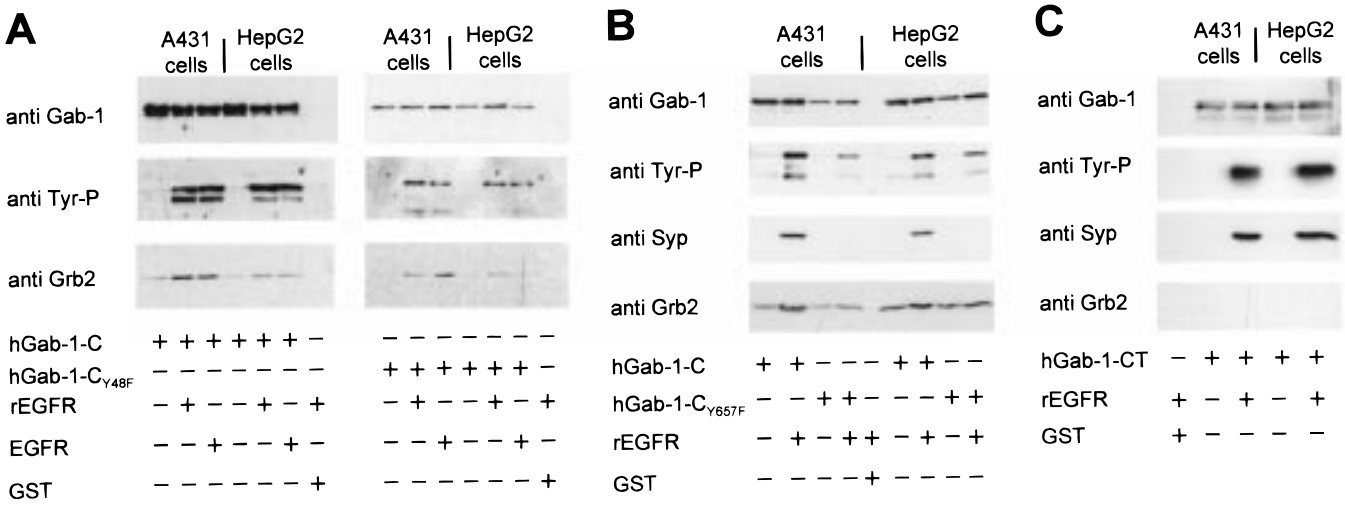


FIGURE 6: Binding of SH2 domain proteins to hGab-1-C in vitro. Phosphorylated and nonphosphorylated wild type hGab-1-C and mutated variants (5 $\mu$g each) or GST were incubated with 1 mg of A431 or HepG2 cell lysates. hGab-1-C with bound proteins was recovered with glutathione beads (see Experimental Procedures). The precipitates were separated by SDS−PAGE and transferred to PVDF membranes. hGab-1, Grb2, and Syp were detected with specific antibodies. Lines below describe the absence (−) or presence (+) of named substances. (A) Comparison of GST pull down assays of wild type hGab-1 (left panel) and hGab-1-$C_{Y48F}$ (right panel). Blots were initially probed with anti-Grb2, and detection by BM chemiluminescence (Boehringer) followed. Subsequently, blots were stripped and reprobed with anti-Gab-1 and anti-αPTyr (RC20). (B) Comparison of GST pull down assays of wild type hGab-1-C and hGab-1-$C_{Y657F}$. The blots were probed with anti-Gab-1, anti-αPTyr (RC20), anti-Syp, and anti-Grb2. (C) GST pull down assays of hGab-1-CT. The blots were probed with anti-Gab-1, anti-αPTyr (RC20), anti-Syp, and anti-Grb2.

ant peptides. It was possible to identify eight different tyrosine residues from hGab-1-C phosphorylated by the EGFR (Table 2). Six of these tyrosine phosphorylation sites are identical with the postulated sites (Figure 5). Tyrosine residues Y447, Y472, and Y619 in YXXM motif are predicted to bind to the SH2 domain in the 85 kDa regulatory subunit (p85) of the PI-3 kinase. Tyrosine phosphorylation site Y657 shows features of a Syp binding site derived from the sequence in IRS-1 that binds to the SH2 domain of Syp. One potential PLC$\gamma$ binding site (Y373) and one which shares features for the SH2 domain of Nck and PLC$\gamma$ (Y406) could also be identified. In contrast, the identified phosphorylation sites, including Y285VFN and Y689VVV, are located in other hydrophobic motifs. Nine additional tyrosine residues may be phosphorylated by the EGFR, given the proximity of an aspartate or glutamate residue. Three of these tyrosine residues are located in motifs that are supposed to bind Grb2, Nck, and PLC$\gamma$. Interestingly, the phosphorylation pattern of EGFR-phosphorylated hGab-1-C displayed a predominant peak during anion exchange HPLC, corresponding to Y657 (50% of incorporated radioactivity) which exhibits features of a Syp binding site. Another identified peptide, which represents tyrosine phosphorylation site Y689, is phosphorylated second-best. These two C-terminal tyrosine residues represent the main tyrosine phosphorylation sites for the EGFR. This correlates with our results. The $K_M$ value of the C-terminal hGab-1 protein is the lowest and similar to the $K_M$ value of the entire protein. The fact that we found no phosphorylation of the tyrosine residue Y48 correlates with the highest $K_M$ value of the N-terminal protein. Phosphorylation of N-terminal tyrosine residues occurred only by using the short N-terminal domain but not in hGab-1-C, which could be caused by the different structure of the proteins. This may be another clue that interaction of hGab-1 with the EGFR appears to be modulated by the structure of the intact hGab-1 protein. These data show that hGab-1 is phosphorylated by EGFR with high specificity and efficiency on tyrosine Y657 and Y689, in vitro. Phosphorylation of hGab-1-C by other receptors might occur on different tyrosine residues. This could modulate the binding of downstream signaling proteins and play a cruical role in the determination of signaling specificity.

*Functional Relevance of Y48 and Y657 Phosphorylation in hGab-1?* To test the functional relevance of our data, GST pull down assays were performed. These experiments exhibit an increase in the extent of binding of Grb2 to EGFR-phosphorylated hGab-1-C. In contrast to that, Tpr-Met-phosphorylated Gab-1 mainly interacts via its SH3 binding motif with Grb2 (*3*). Our data revealed no EGFR phosphorylation of Y48, the postulated Grb2 binding site. There is still the probability that this site is phosphorylated, due to the observed minor unidentified radiolabeled peaks during reversed phase HPLC. By changing Y48 to F48, we proved that this site has no influence on enhancing the binding capability of hGab-1-C.

To determine if binding of Grb2 to hGab-1 was mediated by Syp, pull down assays with hGab-1-$C_{Y657F}$ were performed. In comparison to that of the wild type protein, no significant change in the Grb2 binding pattern was observed. In similar experiments with hGab-1-CT, no binding of Grb2 could be detected. In contrast to that, the phosphorylation-dependent binding of Syp to hGab-1-CT occurred in an identical pattern compared to that of full-length hGab-1. The observed basal hGab-1-C−Grb2 association may be mediated by the middle portion of Gab-1 containing the two SH3 domain binding sites (*1*). These results demonstrate that there is no interaction of Grb2 and hGab-1 in a complex with Syp.

We showed that Y657 in the C-terminal portion of hGab-1, a postulated Syp binding site, is substantially phosphorylated by EGFR and represents the predominant radioactivity peak during anion exchange HPLC. Y657 was identified as the unique Syp binding site in hGab-1. After Y657 is changed to F657, phosphorylated hGab-1 loses its ability to bind Syp without influencing the binding behavior of other signaling proteins such as Grb2. There is a direct, specific, and efficient interaction between hGab-1 and Syp. These results are consistent with the observations of Holgado-Madruga et al. (*1*), who demonstrated a substantial association between Gab-1 and Syp upon stimulation with EGF in vivo. Syp is the major binding partner of Gab-1 when stimulated with EGF. Nearly 15% of the endogenous Syp associates with Gab-1. In contrast, only 1−5% of the endogenous PI-3 kinase and PLC$\gamma$ interacts with Gab-1.

There are three different principles of interaction between phosphorylated tyrosine residues and their binding partners. (1) A single signaling protein is able to bind different phosphorylated tyrosine residues, as shown in the case of the interaction between IRS-1 and PI-3 kinase (*32*). (2) A protein phosphorylated on a tyrosine residue is able to bind different signaling proteins. For example, PI-3 kinase and GAP interact with the same sites on the PDGF receptor (*33*, *34*). (3) The last is phosphorylation of a tyrosine residue that binds solely one signaling protein, as we observed from the interaction between Syp and EGFR phosphorylated hGab-1. This suggests a molecular basis for signaling selectivity.

## ACKNOWLEDGMENT

We thank G. Becker and H. Korte for technical assistance, G. Roth for helpful discussion, and J. Gilette for critical reading of the manuscript. We thank M. Affüpper for the preparation of rEGFR.

## REFERENCES

1. Holgado-Madruga, M., Emlet, D. R., Moscatello, D. K., Godwin, A. K., and Wong, A. J. (1996) *Nature 397*, 560−564.
2. Ong, S. H., Lim, Y. P., Low, B. C., and Guy, G. R. (1997) *Biochem. Biophys. Res. Commun. 238*, 261−266.
3. Fixman, E. D., Holgado-Madruga, M., Nguyen, L., Kamikura, D., Fournier, T., Wong, A. J., and Park, M. (1997) *J. Biol. Chem. 272*, 20167−20172.
4. Holgado-Madruga, M., Moscatello, D. K., Emlet, D. R., Dietrerich, R., and Wong, A. J. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 12419−12424.
5. Daub, H., Wallasch, C., Lankenau, A., Herrlich, A., and Ullrich, A. (1997) *EMBO J. 16*, 7032−7044.
6. Yenush, L., and White, M. F. (1997) *Bioassays 19*, 491−500.
7. Burks, D. J., Pons, S., Towery, H., Smith-Hall, J., Myers, M. G., Jr., Yenush, L., and White, M. F. (1997) *J. Biol. Chem. 272*, 27716−27721.
8. Nguyen, L., Holgado-Madruga, M., Maroun, C., Fixman, E. D., Kamikura, D., Fournier, T., Charest, A., Tremblay, M. L., Wong, A. J., and Park, M. (1997) *J. Biol. Chem. 272*, 20811−20819.
9. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

10. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463−5467.
11. Kasuga, M., White, M. F., and Kahn, C. R. (1985) *Methods Enzymol. 109*, 609−621.
12. Ullrich, A., Coussens, L., Hayflick, J. S., Dull, T. J., Gray, A., Tam, A. W., Lee, J., Yarden, Y., Libermann, T. A., Schlessinger, J., Downward, J., Mayes, E. L. V., Whittle, N., Waterfield, M. D., and Seeburg, P. H. (1984) *Nature 309*, 418−425.
13. Al-Hasani, H., Passlack, W., and Klein, H. W. (1994) *FEBS Lett. 349*, 17−22.
14. Siemeister, G., Al-Hasani, H., Klein, H. W., Kellner, S., Streicher, R., Krone, W., and Müller-Wieland, D. (1995) *J. Biol. Chem. 270*, 4870−4874.
15. Laemmli, U. K. (1970) *Nature 277*, 680−685.
16. Kamps, M. P. (1991) *Methods Enzymol. 201*, 21−27.
17. Rosenfeld, J., Capdevielle, J., Guillemont, J. C., and Ferrara, P. (1992) *Anal. Biochem. 203*, 173−179.
18. Hellmann, U., Wernstedt, C., Gonez, J., and Heldin, C. H. (1995) *Anal. Biochem. 224*, 451−455.
19. Meyer, H. E., Hoffmann-Posorske, E., Donella-Deana, A., and Korte, H. (1991) *Methods Enzymol. 201*, 206−224.
20. Eng, J., McCormack, A. L., and Yates, J. R., III (1994) *Am. Soc. Mass Spectrom. 5*, 976−989.
21. Yates, J. R., III, Eng, J., McCormack, A., and Schieltz, D. (1995) *Anal. Chem. 67*, 1426−1436.
22. Towbin, H., Staehlin, T., and Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4350−4354.
23. Al-Hasani, H., Eisermann, B., Tennagels, N., Magg, C., Passlack, W., Koenen, M., Müller-Wieland, D., Meyer, H. E., and Klein, H. W. (1997) *FEBS Lett. 400*, 65−70.
24. Sun, X. J., Rothenberg, P., Kahn, C. R., Backer, J. M., Araki, E., Wilden, P. A., Cahill, D. A., Goldstein, B. J., and White, M. F. (1991) *Nature 352*, 73−77.
25. Sun, X. J., Wang, L.-M., Zhang, Y., Yenush, L., Myers, M. G., Jr., Glasheen, E., Lane, W. S., Pierce, J. H., and White, M. F. (1995) *Nature 377*, 173−177.
26. Lavan, B. E., Lane, W. S., and Lienhard, G. E. (1997) *J. Biol. Chem. 272*, 11439−11443.
27. Lavan, B. E., Fantin, V. R., Chang, E. T., Lane, W. S., Keller, S. R., and Lienhard, G. E. (1997) *J. Biol. Chem. 272*, 21403−21407.
28. Myers, M. G., Jr., Cheatham, B., Fisher, T. L., Jachna, B. R., Kahn, C. R., Backer, J. M., and White, M. F. (1996) *Ann. N.Y. Acad. Sci. 766*, 369−387.
29. Weidner, K. M., Cesare, S. D., Sachs, M., Brinkmann, V., Behrens, J., and Birchmeier, W. (1996) *Nature 384*, 173−176.
30. Myers, M. G., Jr., Sun, X. J., Cheatham, B., Jachna, B. R., Glasheen, E. M., Backer, J. M., and White, M. F. (1993) *Endocrinology 132*, 1421−1430.
31. Shoelson, S. E., Chatterjee, S., Chaudhuri, M., and White, M. F. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 2027−2031.
32. Sun, X. J., Crimmins, D. L., Myers, M. G., Jr., Miralpeix, M., and White, M. F. (1993) *Mol. Cell. Biol. 13*, 7418−7428.
33. Kazlauskas, A., and Cooper, J. A. (1990) *EMBO J. 9*, 3279−3286.
34. Kazlauskas, A., Ellis, C., Pawson, T., and Cooper, J. A. (1990) *Science 247*, 1578−1581.

BI9818265