Phosphorylation of Grb10 by Mitogen-Activated Protein Kinase: Identification of Ser^{150} and Ser^{476} of Human Grb10ζ as Major Phosphorylation Sites†

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ABSTRACT: Grb10 is a Src-homology 2 (SH2) and Pleckstrin-homology (PH) domain-containing protein that binds to several autophosphorylated receptor tyrosine kinases including the insulin receptor (IR). Our previous studies showed that Grb10 underwent insulin-stimulated serine phosphorylation, yet the kinase(s) responsible for phosphorylation and the sites of the phosphorylation remain unknown. In this report, we show that Grb10 is a direct substrate of the p42/44 mitogen-activated protein kinase (MAPK). In addition, we found that inhibition of the MAPK signaling pathway reduced Grb10 phosphorylation in cells. Using site-directed mutagenesis, phosphopeptide mapping, and capillary HPLC−electrospray−tandem mass spectrometry analysis, we identified Ser^{150}, Ser^{418}, and Ser^{476} of human Grb10ζ as MAPK-mediated in vitro phosphorylation sites. In vivo labeling and two-dimensional phosphopeptide mapping studies revealed that Ser^{150} and Ser^{476} of human Grb10ζ are phosphorylated in intact cells. Replacing Ser^{150} and Ser^{476} with alanines reduced the inhibitory effect of human Grb10ζ on insulin-stimulated IRS1 tyrosine phosphorylation. Taken together, our findings suggest that phosphorylation of the adaptor protein may provide a feedback inhibitory mechanism by which Grb10 regulates insulin signaling.

Insulin action is initiated by binding of the hormone to its membrane receptor in target cells. Upon insulin binding, the tyrosine kinase activity of the β-subunit of the insulin receptor (IR)[1] becomes activated, resulting in the subsequent phosphorylation of downstream signaling molecules such as IRS-1 and IRS-2, which leads to activation of the phosphatidylinositol (PI) 3-kinase signaling pathway. Binding of insulin also results in autophosphorylation of the insulin receptor, leading to the association of the adaptor protein Shc, which serves to activate the mitogen-activated protein kinase (MAPK) signaling pathway. Through both the PI 3-kinase and MAPK pathways, insulin stimulates a variety of cellular responses including glucose uptake, cellular growth, metabolism, and differentiation (*1*).

Grb10 is a SH2 and PH-domain-containing adaptor protein that binds to tyrosine-phosphorylated insulin and insulin-like growth factor 1 (IGF1) receptors in response to insulin or IGF1 stimulation (*2*, *3*). There is some controversy on whether Grb10 is a positive or negative regulator of insulin signaling (*4*−*7*). Two recent studies showed that reducing the expression levels of endogenous Grb10 led to increased growth in mice (*8*) and enhanced insulin signaling in cells (*9*), suggesting that endogenous Grb10 may act as a negative regulator of both growth and insulin signaling.

Grb10 has been shown to be phosphorylated on both serine (*10*, *11*) and tyrosine residues (*12*, *13*). Although Tec, Src, and Fyn have been identified as the kinases responsible for tyrosine phosphorylation of Grb10 (*12*, *13*), the kinase(s) that mediate(s) Grb10 serine phosphorylation remain(s) uncharacterized. In the basal state, overexpressed human Grb10 possesses a differential gel mobility which undergoes an upward shift to the higher molecular weight band upon insulin stimulation (*10*). Similar results were reported for mouse Grb10 stimulated with EGF, FGF, and PDGF (*11*). The Grb10 differential gel mobility represents multiple phosphorylation states of the protein since phosphatase treatment abolishes the higher molecular weight bands (*10*). In addition to phosphatase treatment, the apparent gel mobility shift of Grb10 was inhibited by treating cells with either the PI 3-kinase inhibitor LY294002 or the MAPK pathway inhibitor PD98059 (*10*). These results suggest that kinases within both the PI 3-kinase and the MAPK signaling pathways may phosphorylate Grb10.

In this study, we show that Grb10 is a direct substrate of MAPK. In vivo labeling and two-dimensional phosphopeptide mapping studies revealed Ser^{150} and Ser^{476} of human

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[1] Abbreviations: IR, insulin receptor; IGF1R, insulin-like growth factor 1 receptor; IRS, insulin receptor substrate; CHO, Chinese hamster ovary; SH2, Src-homology 2; PH, Pleckstrin homology; BPS, between the PH and SH2; PI 3-kinase, phosphatidylinositol 3-kinase; ERK, extracellular signal-related kinase; MAPK, mitogen-activated protein kinase; MALDI-TOF/MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; HPLC-ESI/MS/MS, capillary HPLC−electrospray−tandem mass spectrometry.

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Grb10ζ as two in vivo phosphorylation sites. Replacing Ser^{150} and Ser^{476} with alanines reduced the inhibitory effect of human Grb10ζ on insulin-stimulated IRS1 tyrosine phosphorylation. Taken together, our findings reveal that Grb10 is a direct substrate of MAPK and suggest that phosphorylation of the adaptor protein at Ser^{150} and Ser^{476} may provide a feedback inhibitory mechanism by which Grb10 regulates insulin signaling.

MATERIALS AND METHODS

Cell Lines and Reagents. A Chinese hamster ovary cell line overexpressing the insulin receptor (CHO/IR) and cDNA encoding human full-length human Grb10ζ have been described previously (*10*). Biochemical reagents were obtained from the following sources: HA monoclonal antibody, Covance; LY294002, Sigma; PD98059, Calbiochem; secondary antibodies conjugated to alkaline phosphatase and horseradish peroxidase, Promega; protein G−Sepharose beads, Amersham-Pharmacia Biotech; glutathione−Sepharose beads, Sigma; C_{18} ZipTips, Millipore; TPCK-treated trypsin, Worthington Biochemical Corp.; antibody to phosphotyrosine, Transduction Laboratories.

Generation of Truncation Mutants of Full-Length Human Grb10. A cDNA encoding amino acids 36−594 of human Grb10ζ (*10*) was generated by PCR using full-length human Grb10ζ cDNA as a template (for nomenclature of Grb10 isoforms, please see refs *2* and *3*). The PCR primers used were FW, 5′-CGGAATTCCCCGCACAGTCTGAC-3′, and RV, 5′-CTGCGTCGACCGCTTCTTCACTC-3′. Using a similar approach, we also generated expression vectors encoding a carboxyl-terminal truncated mutant of human Grb10ζ [residues 36−473; Grb10 (ΔSH2)] and the C-terminal of human Grb10ζ [residues 410−594; Grb10 (BPS + SH2)] using the following PCR primer pairs: 5′-CGGAATTCCCCGCACAGTCTGAC-3′, 5′-GCTGTCGAC-CTAGGATGTTCATCCG-3′ and 5′-GCGAATTCCCTCAG-CAGAGGAAG-3′, 5′-CTGCGTCGACCGCTTCTTCACTC-3′, respectively. After restriction digestion, the cDNA fragments were subcloned into the GST fusion protein expression plasmid pGEX-4-T1, in frame at their N-termini with a sequence encoding GST (Pharmacia Biotech).

Construction of Plasmids and Site-Directed Mutagenesis. Human $Grb10\zeta^{S104G}$, $Grb10\zeta^{S150I}$, $Grb10\zeta^{S418A}$, $Grb10\zeta^{S418A/S421A/T422A}$, $Grb10\zeta^{S476A}$, $Grb10\zeta^{S150I/S476A}$, and $Grb10\zeta^{S150D/S476D}$ were generated by single-stranded site-directed mutagenesis according to the protocol as described by Kunkel (*14*) using customized primers. The subcloning of these cDNAs into the mammalian expression vector pBEX, in frame with the HA tag, was performed according to the previously described protocol (*10*). The generation of cDNA encoding Myc-tagged full-length mutants of Grb10 was accomplished as described previously (*7*). All site-directed mutagenesis products were confirmed by restriction mapping and DNA sequencing. The construct for IRS-1 (*7*) was described previously.

Expression and Purification of Proteins in Bacterial Cells. BL21(DE3) cells containing plasmids encoding GST fused to either human Grb10ζ, Grb10(ΔSH2), or Grb10(BPS+SH2) were grown in LB medium containing ampicillin. Expression of proteins was induced by the addition of 1 mM isopropyl β-D-thiogalactoside (IPTG) for 3.5 h at 30 °C. Cells were harvested by centrifugation at 5000*g* for 10 min and maintained at −80 °C for 10 min. The cells were then resuspended in ice-cold buffer C (50 mM Tris-HCl, pH 7.5, 50 mM KCl, 1 mM dithiothreitol, 5 mM EDTA, 1 mM phenylmethanesulfonyl fluoride, 0.1% Triton X-100, and 1 mg/mL lysozyme), followed by incubation on ice for 30 min. The solution was sonicated and clarified by centrifugation at 14000*g* for 15 min. The proteins were purified by affinity chromatography using glutathione−Sepharose beads.

Cell Culture, Immunoprecipitation, and Western Blot. CHO/IR cells were grown in Ham's F-12 medium (Life Technologies) supplemented with 10% newborn calf serum and 1% penicillin/streptomycin. Transfections of CHO/IR cells were performed in 100 mm plates with 10 μg of total recombinant plasmid, using LipofectAMINE reagent according to the manufacturer's protocol (InVitrogen). Twenty-four hours after transfection, cells were lysed in 300 μL of buffer A (50 mM Hepes, pH 7.6, 150 mM NaCl, 1% Triton X-100, 10 mM NaF, 20 mM sodium pyrophosphate, 20 mM β-glycerol phosphate, 1 mM sodium orthovanadate, 10 μg/mL leupeptin, 10 μg/mL aprotinin, and 1 mM phenylmethanesulfonyl fluoride). The homogenate was centrifuged (14000*g*, 4 °C, 10 min), and the supernatants were incubated with specific antibodies bound to protein G beads overnight at 4 °C with gentle rotation. After incubation, immunoprecipitates were washed extensively with ice-cold buffer B (50 mM Hepes, pH 7.6, 150 mM NaCl, and 0.1% Triton X-100). Proteins bound to the beads were eluted by heating at 95 °C for 4 min in SDS−PAGE sample loading buffer. The eluted proteins were separated by SDS−PAGE, transferred to a nitrocellulose membrane, and detected with specific antibodies.

In Vitro Phosphorylation of Grb10 by MAPK. Recombinant wild-type p42 MAPK (ERK2) was activated in vitro by incubation at 30 °C for 1 h in MEK kinase buffer (20 mM Hepes, pH 7.5, 20 mM $MgCl_2$, 0.1 mg/mL BSA, and 2 mM ATP) in the presence of activated MEK1 [ERK2 and activated MEK1, which were purified to near homogeneity by nickel chelate chromatography, were generous gifts from Dr. J. E. Ferrell (*15*−*19*)]. HA-tagged Grb10 proteins transiently expressed in CHO/IR cells were immunoprecipitated with anti-HA antibody adsorbed onto protein G−agarose beads. After overnight incubation at 4 °C, the beads were collected and washed twice with ice-cold buffer B and once with ice-cold MEK kinase buffer. Phosphorylation of Grb10 by MAPK was carried out at 30 °C for 30 min in the presence of MEK kinase buffer (final volume 30 μL), 2 μCi of [γ-^{32}P]ATP (PerkinElmer Life Sciences), and activated MAPK. The reaction was stopped by the addition of SDS sample buffer and heating at 95 °C for 4 min. The proteins were separated by SDS−PAGE using 10% (w/v) polyacrylamide gels and transferred to a nitrocellulose membrane, and the phosphorylated Grb10 was visualized by autoradiography.

In Vivo ^{32}P Labeling and Phosphopeptide Mapping of Grb10ζ. In vivo ^{32}P metabolic labeling and phosphopeptide mapping studies of Grb10 were carried out using similar protocols as described previously (*20*).

Mass Spectrometry. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF/MS) was conducted on an Applied Biosystems Voyager-DE STR. For analysis of tryptic digests of Grb10 and extracts of spots from 2-D TLC plates, 2,5-dihydroxybenzoic acid (DHB) was

employed as the matrix, and the instrument was operated in the reflectron mode with positive ion detection. MALDI-TOF spectra were generated by averaging the results from 100 laser shots, with spectral processing by smoothing (five points) and noise reduction. The peptide mass maps produced by MALDI-TOF/MS of tryptic digests of Grb10 were searched against published databases by means of the MS-Fit module of Protein Prospector (http://prospector.ucsf.edu/) and MASCOT (Matrix Science, http://www.matrixscience.com) in order to confirm the presence of the target protein. HPLC−electrospray ionization−tandem mass spectrometry (HPLC-ESI/MS/MS) was performed on a Thermo Finnigan LCQ, which has been adapted for microspray ionization. On-line HPLC separations were accomplished with a Michrom BioResources MAGIC 2002 micro-HPLC as follows: column, PicoFrit (New Objective; 75 μm i.d.) packed to 10 cm with C18 adsorbent (Vydac; 218MS 5 μm, 300 Å); mobile phase A, 0.5% acetic acid/0.005% TFA; mobile phase B, 90% acetonitrile (ACN)/0.5% acetic acid/0.005% TFA; linear gradient of 2−72% B in 30 min; flow rate, 0.4 μL/min. For initial analysis of tryptic digests, a data-dependent acquisition protocol was utilized in which the four most intense ions in each survey scan were sequentially fragmented in the ion trap by collision-induced dissociation (CID) using an isolation width of 2.5 and a relative collision energy of 35%. The uninterpreted MS/MS spectra obtained by this approach were analyzed by the SEQUEST component of the LCQ software and by MASCOT (Matrix Science). For detection of selected Grb10 peptides ("targeted MS/MS"), the appropriate 1+ and 2+ ions were specifically trapped and fragmented. Assignment of all MS/MS fragments was verified by comparison with the predicted ions generated in silico by GPMAW (Lighthouse Data).

Statistical Significance. Results are expressed as the mean ± SEM. Differences between the groups were examined for statistical significance using analysis of variance (ANOVA).

RESULTS

Grb10 Undergoes Insulin-Stimulated and PI 3-Kinase/MAPK-Dependent Phosphorylation in Cells. We previously showed that treatment of cells with insulin resulted in a gel mobility shift of Grb10, which was abolished by incubation of Grb10 with potato alkaline phosphatase (PAP) (*10*). Grb10 differential gel mobility was also prevented by treatment of cells with either the PI 3-kinase inhibitor LY294002 or the MEK1 inhibitor PD98059 (*10*). Together, these findings suggest that Grb10 is a potential substrate for kinases involved in both the PI 3-kinase and MAPK signaling pathways.

To provide direct evidence that Grb10 is phosphorylated by kinases in the PI 3-kinase and the MAPK pathways, metabolic labeling experiments were performed on CHO/IR cells transiently overexpressing human Grb10ζ. In agreement with previous reports (*10*), insulin stimulation resulted in an increase of Grb10 phosphorylation, as visualized by incorporation of ^{32}P into the protein (Figure 1A, lanes 1 vs 2 and Figure 1B). Inhibition of the PI 3-kinase pathway with LY294002 resulted in a decrease of Grb10 phosphorylation, in both basal and insulin-stimulated conditions (Figure 1A, lanes 5 and 6 vs lanes 1 and 2). Treatment of cells with the MEK1 inhibitor PD98059 also resulted in a decrease, albeit

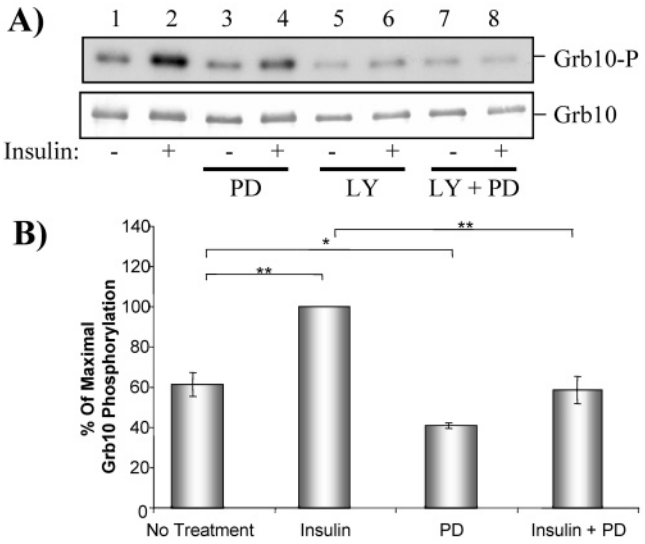

FIGURE 1: PD98059 and LY294002 inhibit the basal and insulin-stimulated phosphorylation of Grb10. (A) CHO/IR cells transiently expressing human Grb10ζ were labeled with [^{32}P]orthophosphate and left untreated (lanes 1 and 2) or treated with PD98059 (50 μM, lanes 3 and 4), LY294002 (50 μM, lanes 5 and 6), or LY294002 and PD98059 (lanes 7 and 8) for 1 h and then treated with (+) or without (−) insulin (10^{-8} M) for 5 min. Grb10ζ was immunoprecipitated with antibody against the HA tag. The phosphorylation and expression of Grb10ζ were visualized by autoradiography (upper panel) or by Western blot using an anti-HA antibody (lower panel). Data are representative of four individual experiments. (B) Quantitative analysis of the effect of PD98059 on Grb10 phosphorylation in cells. Grb10 phosphorylation was quantified using Scion Image and normalized to Grb10 expression levels. The mean fold stimulation is expressed as a percentage of the maximum ± SE ($n = 4$). Key: *, $P < 0.05$; **, $P < 0.01$.

to a lesser extent, of Grb10 phosphorylation in both the basal and insulin-stimulated states (Figure 1A, lanes 3 and 4 vs lanes 1, 2, 5, and 6, and Figure 1B). Inhibition of both the MAPK and PI 3-kinase pathways together significantly decreased Grb10 phosphorylation (Figure 1A, lanes 7 and 8 vs lanes 1 and 2). Taken together, these results suggest that Grb10 is a substrate of kinases involved in both the PI 3-kinase and MAPK pathways.

Grb10 Is a Direct Substrate of MAPK in Vitro. Examination of the Grb10 sequence revealed several potential MAPK phosphorylation sites [(P)-X-S/T-P (*21*) or S/T-P (*22*)]. To test whether Grb10 is a direct substrate for MAP kinase, we carried out in vitro phosphorylation studies. HA-tagged human Grb10β or Grb10ζ (*2*) was transiently expressed in CHO/IR cells, immunoprecipitated with antibody to the HA tag, and incubated in vitro with bacterially expressed, purified, and activated p42 MAPK in the presence of [γ-^{32}P]-ATP. Incubation with MAPK resulted in phosphorylation of both Grb10 isoforms (Figure 2A, lanes 3 and 4 vs lane 2). A time course experiment revealed that phosphorylation of Grb10 by MAPK could be observed as early as 5 min and persisted up to 30 min (data not shown). No phosphorylation was observed when Grb10 isoforms were incubated with activated MEK1 alone (Figure 2A, lane 1). Taken together with the finding that Grb10 phosphorylation is inhibited by the MEK1 inhibitor PD98059 (Figure 1), these results suggest that Grb10 is a direct substrate of MAPK.

Map the Regions of Grb10 Phosphorylated by MAPK in Vitro. To identify the MAPK-mediated Grb10 phosphorylation site(s), we first carried out studies to map the region-

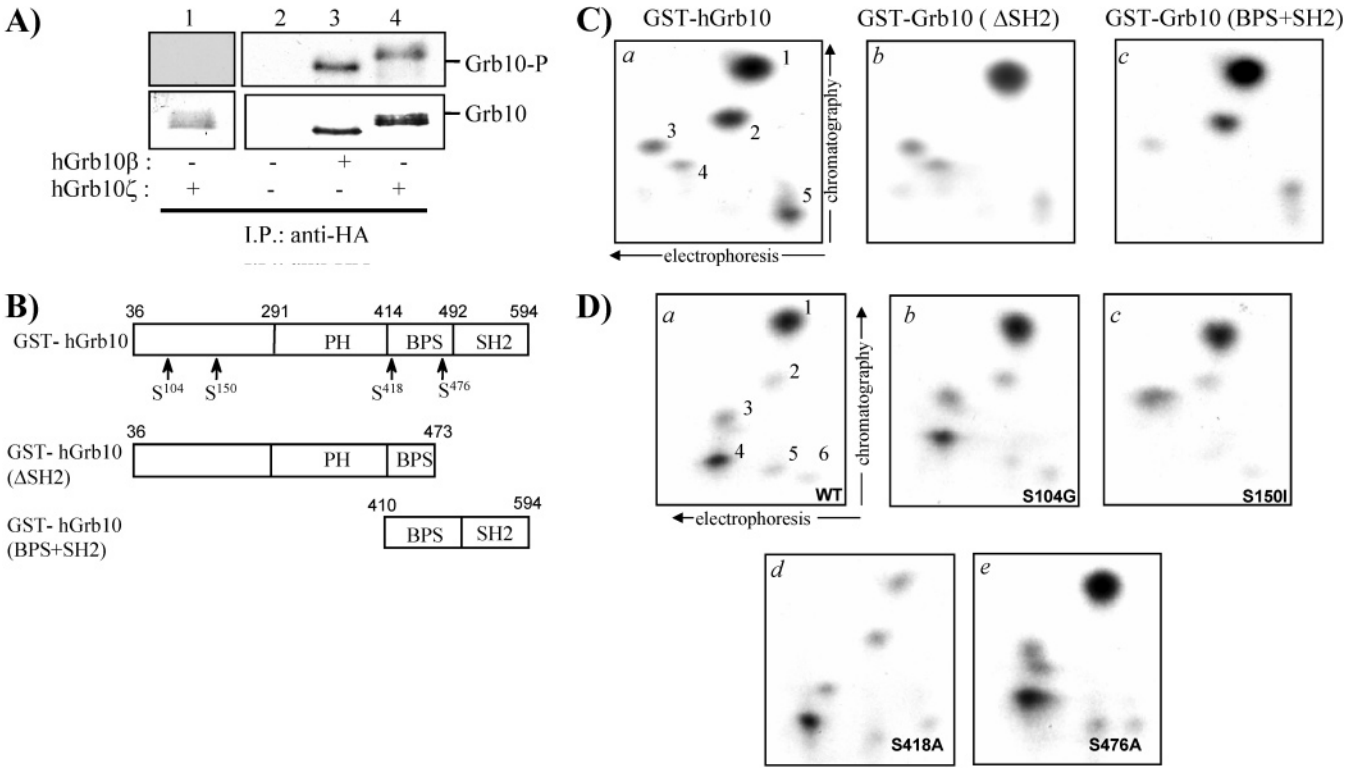

FIGURE 2: Identification of Grb10 as a substrate of MAPK. (A) Phosphorylation of the PH-domain truncated human Grb10β and full-length human Grb10ζ isoforms by MAPK in vitro. CHO/IR cells were transiently transected with either empty vector (lane 2), PH-domain truncated human Grb10β (lane 3), or full-length human Grb10ζ (lanes 1 and 4). The proteins were immunoprecipitated with antibody to the HA tag and incubated with constitutively active MEK1 alone (lane 1) or MEK1 together with activated p42 MAPK (ERK2) in the presence of [γ-^{32}P]ATP. Grb10 phosphorylation and expression were determined by autoradiography (upper panel) or by Western blot with anti-HA antibody (bottom panel), respectively. Data are representative of at least three individual experiments. (B) Diagrams of full-length and various truncations of human Grb10ζ. Truncation (C) and point mutants (D) of Grb10 were in vitro phosphorylated by MAPK and analyzed by two-dimensional phosphopeptide mapping.

(s) on Grb10 that contain potential MAPK phosphorylation sites. Several GST fusion proteins representing different regions of human Grb10ζ (Figure 2B) were expressed in bacterial cells, purified, and phosphorylated by activated MAPK in vitro. The phosphorylated truncated Grb10 proteins were then subjected to trypsin digestion and two-dimensional phosphopeptide analyses. In vitro phosphorylation of GST-Grb10ζ resulted in five phosphopeptides (Figure 2C, panel a). Phosphopeptide mapping of Grb10 (ΔSH2) revealed the loss of phosphopeptide 2, suggesting that one of the major MAPK-catalyzed phosphorylation sites resided within amino acids 474−594 of human Grb10ζ (Figure 2C, panel b). Phosphopeptide mapping of Grb10 (BPS + SH2) revealed the loss of phosphopeptide 4, indicating that one of the major MAPK-catalyzed phosphorylation sites on Grb10ζ is present between amino acids 36−409 (Figure 2C, panel c).

Identification of Ser^{150} and Ser^{476} of Human Grb10ζ as MAPK-Catalyzed Phosphorylation Sites in Vitro. Examination of the sequence of human Grb10ζ (*2*) revealed several potential MAPK phosphorylation sites [P-X-S/T-P (*21*) or S/T-P (*22*)]: T^{23}-P, S^{104}-P, P -G-S^{150}-P, T^{155}-P, S^{418}-P, T^{422}-P, and S^{476}-P (Figure 2B). Previous studies on both human Grb10 (*10*) and mouse Grb10 (*11*) found that Grb10 phosphorylation in cells occurs predominantly on serine residues. To test whether the potential MAPK consensus phosphorylation serine residues on Grb10 were phosphorylated by MAPK in vitro, site-directed mutagenesis was performed to generate HA-tagged S104G, S150I, S418A, and S476A Grb10 mutants. The HA-tagged wild-type or mutant Grb10ζ proteins were transiently expressed in CHO/IR cells, immunoprecipitated, and phosphorylated by activated MAPK in vitro in the presence of [γ-^{32}P]ATP. No major differences were observed between the phosphopeptide maps of the S104G mutant and wild-type Grb10 (Figure 2D, panel a vs panel b). In contrast, phosphopeptide mapping of the S150I and S476A mutants revealed the loss of phosphopeptide 4 and phosphopeptide 2, respectively (Figure 2D, panels c and e vs panel a). These results identified Ser^{150} and Ser^{476} of human Grb10ζ as major MAPK-catalyzed phosphorylation sites in vitro. While replacing Ser^{418} with alanine did not result in the net loss of any phosphopeptides, this mutation did lead to a marked reduction in phosphorylation of phosphopeptide 1 as compared to wild-type hGrb10ζ (Figure 2D, panel d). One possible explanation for this result is that the Ser^{418}-containing tryptic peptide possesses a second MAPK consensus phosphorylation site, Thr^{422} (Figure 3). Therefore, Thr^{422} in the Ser^{418}-containing peptide may act as a compensatory phosphorylation site on Grb10 upon mutation of Ser^{418}, resulting in the appearance of phosphopeptide 1 on the two-dimensional phosphopeptide map of Grb10^{S418A} phosphorylated by MAPK in vitro.

Identification of Ser^{418} of Human Grb10ζ as an in Vitro MAPK Phosphorylation Site. To elucidate the identity of phosphopeptide 1 of the in vitro phosphorylated wild-type Grb10 (Figure 3, inset), the peptides in spot 1 were eluted from the thin-layer chromatography plate and analyzed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF/MS). A peptide with a molecular

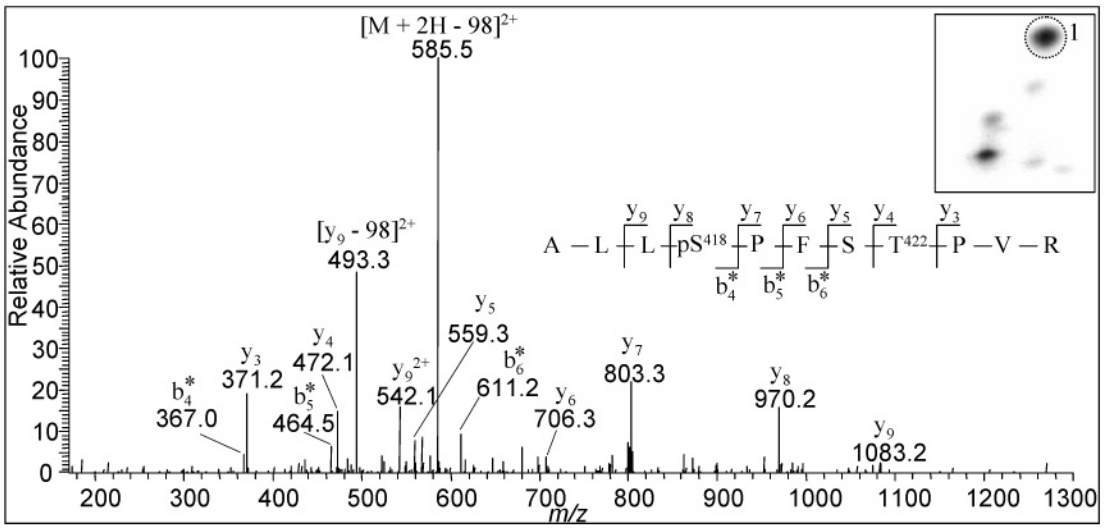

FIGURE 3: Identification of Ser[418] of human Grb10ζ as an in vitro MAPK phosphorylation site by HPLC-ESI/MS/MS. In vitro phosphorylation of wild-type Grb10ζ was performed as described in Figure 2C in the absence of [γ-^{32}P]ATP. Grb10 protein bands were excised from the membrane and digested with trypsin, and the peptides were analyzed by capillary HPLC-ESI/MS/MS.

mass of 1266 Da, consistent with the mass of phosphorylated Grb10ζ-415−425, was detected (data not shown). Further analysis of this peptide by capillary HPLC−electrospray−tandem mass spectrometry (HPLC-ESI/MS/MS) resulted in a spectrum unambiguously confirming the presence of phosphorylated-Grb10-415−425 (ALLpSPFSTPVR; 1266 Da). The spectrum verified that Ser[418], but not Ser[421] or Thr[422], was the specific site of phosphorylation (data not shown). To confirm this finding, GST-Grb10ζ was phosphorylated by MAPK in vitro in the presence of unlabeled ATP. Grb10 was separated by SDS−PAGE, and proteins on the SDS−PAGE gel were detected by Coomassie blue staining. The band corresponding to Grb10 was excised from the gel and subjected to in-gel tryptic digestion, and the resulting peptides were analyzed by targeted HPLC-ESI/MS/MS. The spectrum was produced by collision-induced dissociation (CID) of the ion at *m*/*z* 634.4, which represents a doubly charged ion of the phosphorylated Grb10ζ-415−425 peptide. The fragment characteristic of a phosphopeptide representing the loss of phosphoric acid from the 2+ ion can be seen at *m*/*z* 585.5. The C-terminal fragment series (y_3−y_9) and the N-terminal fragments b_4−b_6 definitively place the phosphate substituent on Ser[418] (Figure 3).

Ser[150] and Ser[476] of Human Grb10ζ Are Phosphorylated in Intact Cells. To test whether Ser[150], Ser[418], and Ser[476] of Grb10ζ were phosphorylated in intact cells, in vivo metabolic labeling experiments were carried out. CHO/IR cells expressing either wild-type or point mutants of hGrb10ζ were incubated with [^{32}P]orthophosphate, and the labeled Grb10 proteins were subsequently isolated and subjected to two-dimensional phosphopeptide mapping analysis. The two-dimensional phosphopeptide maps of basal and insulin-stimulated hGrb10ζ resulted in an equal number of phosphopeptides, but the intensity of several phosphopeptides was increased after insulin stimulation (Figure 4, panel a vs panel b). In vivo labeling and two-dimensional phosphopeptide analysis of the Grb10 triple mutant (Grb10$^{S418A/S421A/T422A}$) revealed no change of the phosphopeptide map compared to wild-type Grb10, indicating that Ser[418] was not phosphorylated in intact CHO/IR cells (data not shown). Mutation of Ser[150] led to a loss of phosphopeptide 2 (Figure 4, panel c vs panels a and b), and mutation of Ser[476] led to a loss of phosphopeptide 3 (Figure 4, panel d vs panels a and b) while the double mutant hGrb10$^{S150I/S476A}$ lacks both phosphopeptide 2 and 3 (Figure 4, panel e vs panels a and b). Interestingly, mutation of Ser[476] also resulted in a loss of the minor phosphopeptide 4 (Figure 4, panel d vs panels a and b). One possible explanation for this observation may be that tryptic digestion of Grb10 may have resulted in multiple Ser[476]-containing phosphopeptides due to incomplete digestion at either Arg[463] or Arg[468] within the Ser[476]-containing tryptic peptide. In agreement with this possibility, we have recently observed the appearance of multiple phosphopeptides corresponding to one phosphorylation site in our phosphopeptide mapping studies of PDK1 autophosphorylation at Thr[516] (*20*).

Mutations at Ser[150] and Ser[476] Reduce the Inhibitory Effect of hGrb10ζ on Insulin Signaling. We have previously found that overexpression of Grb10 inhibits insulin-stimulated phosphorylation of IRS-1 (*7*). To determine the potential role of Grb10 phosphorylation, we compared the effect of wild type and the phosphorylation site mutant of Grb10 on insulin-stimulated tyrosine phosphorylation of IRS-1. To avoid the possibility that overexpression of Grb10 may mask subtle differences between wild-type and mutant Grb10 in their ability to inhibit insulin signaling, we performed dose curve experiments to establish an optimal condition at which different effects between wild-type and mutant Grb10 on insulin-stimulated IRS-1 tyrosine phosphorylation could be detected (data not shown). We found that the hGrb10ζ$^{S150I/S476A}$ mutant had a reduced inhibitory effect on insulin-stimulated phosphorylation of IRS-1 compared to wild-type hGrb10ζ (Figure 5A, lane 4 vs lane 3). Quantification analysis showed that hGrb10ζ$^{S150I/S476A}$ inhibits IRS-1 phosphorylation less efficiently than wild-type hGrb10ζ (Figure 5B). These results suggest that serine phosphorylation of Grb10 may play a role in regulating Grb10/IR interaction.

DISCUSSION

Grb10 binds to the tyrosine-phosphorylated insulin receptor in response to insulin stimulation, and this association

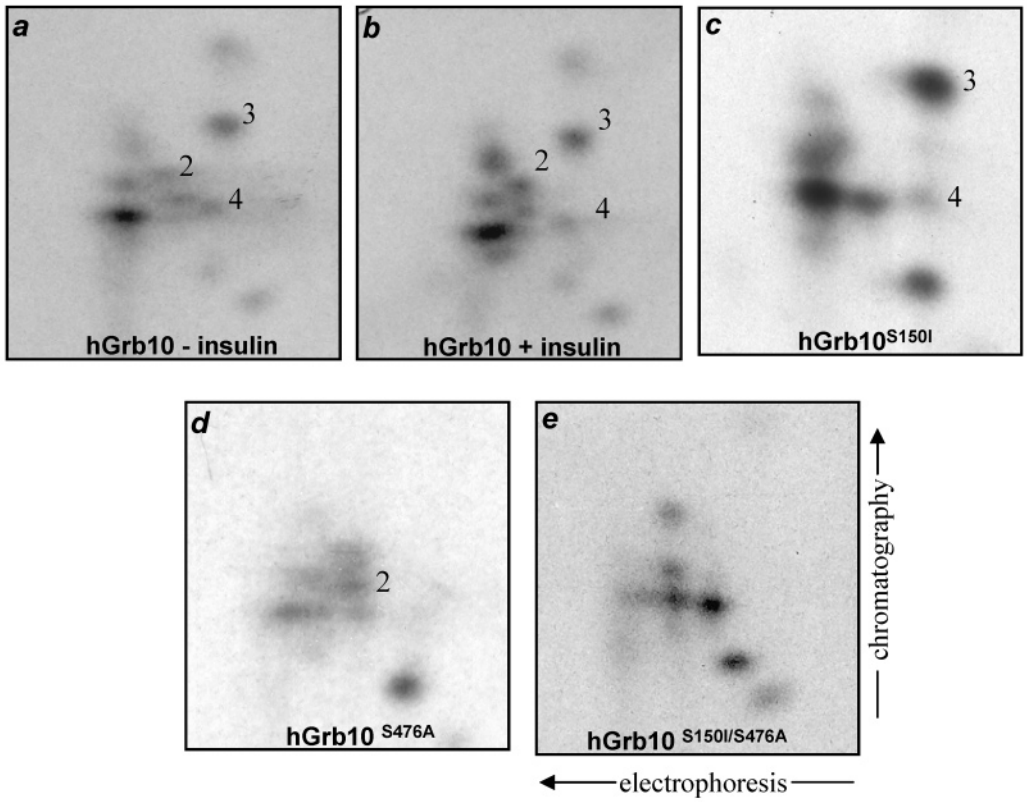

FIGURE 4: Identification of Ser[150] and Ser[476] of hGrb10ζ as phosphorylation sites in cells. CHO/IR cells transiently expressing either wild-type or mutant Grb10ζ were labeled with [^{32}P]orthophosphate and left either untreated (a, c, d, e) or treated with 10^{-8} M insulin for 5 min (b). Grb10 was immunoprecipitated with antibody against the HA tag, and its phosphorylation was analyzed by two-dimensional phosphopeptide analysis.

has been shown to regulate insulin-stimulated PI 3-kinase and MAPK signaling. We and others have previously shown that, in cells, Grb10 is phosphorylated on both serine (*10*, *11*) and tyrosine residues (*12*, *13*). Tyrosine phosphorylation of Grb10 has been shown to negatively regulate its binding to the IR (*13*); however, the kinases that catalyze Grb10 serine phosphorylation and the roles of serine phosphorylation in regulating Grb10 function have not been characterized.

In this study, we show that hGrb10ζ is a direct substrate of MAPK and is phosphorylated by the p44/42 MAPK in vitro at Ser[150], Ser[418], and Ser[476]. We also demonstrated that Ser[150] and Ser[476] of hGrb10ζ are phosphorylated in intact cells. Together with the finding that Grb10 phosphorylation in cells is inhibited by PD98059, a specific inhibitor of the MAPK upstream kinase (MEK) (Figure 1), our results suggest that Grb10 is a physiologically relevant substrate of the p44/42 MAPK.

In addition to being phosphorylated by MAPK, our results also show that inhibition of the PI 3-kinase pathway blocks Grb10 phosphorylation. Phosphorylation by kinases within both the MAPK and PI 3-kinase pathways has been reported for other insulin signaling molecules such as the 90 kDa ribosomal S6 kinase 1 (RSK1) protein, which acts as a substrate for both MAPK and phosphoinositide-dependent kinase 1 (PDK1) (*23*). During the revision of this paper, a study reported the identification of Akt as a kinase within the PI 3-kinase signaling pathway capable of phosphorylating Grb10 (*24*). Our unpublished studies revealed that Grb10 is a direct substrate of PKCζ, another serine/threonine kinase downstream of PI 3-kinase (K. L. Wick and F. Liu, unpublished studies). This result is in agreement with a recent finding that another Grb7/10/14 family member, Grb14, is a substrate for PKCζ (*25*). Further studies aimed at determining whether Grb10 is a physiological substrate of PKCζ are currently in progress.

Interestingly, treatment of cells with either LY294002 or PD98059 resulted in a generic decrease of all the phosphorylation sites (data not shown), suggesting the possibility that phosphorylation at either the ERK and/or the PI 3-kinase-dependent phosphorylation sites may act as a "primer" for subsequent phosphorylation at other sites. Hierarchal phosphorylation has been observed for several other proteins, including glycogen synthase and the transcription factor FOXO1. Glycogen synthase becomes phosphorylated at Ser[7], allowing for the subsequent phosphorylation of Ser[10] by casein kinase-1 (*26*). FOXO1 undergoes phosphorylation at Ser[319] by protein kinase B (Akt/PKB), which permits sequential phosphorylation at Ser[322] and Ser[325] (*27*). FOXO1 is also phosphorylated at Ser[253], which is a prerequisite for the phosphorylation of Thr[24] and Ser[316] (*28*). However, whether Grb10 undergoes hierarchal phosphorylation requires further identification of the remaining Grb10 phosphorylation sites and the kinases responsible for the phosphorylation.

The finding that Grb10 is a direct substrate of the p42 MAPK is of interest since Grb10 has been shown to inhibit insulin-stimulated activation of both the MAPK (*9*, *29*) and PI 3-kinase (*7*) pathways by preventing IR substrate phosphorylation. Thus, it is possible that phosphorylation of Grb10 by MAPK may provide a feedback mechanism to regulate insulin signaling. In agreement with this, we found that expression of the hGrb10ζ$^{S150I/S476A}$ double mutant

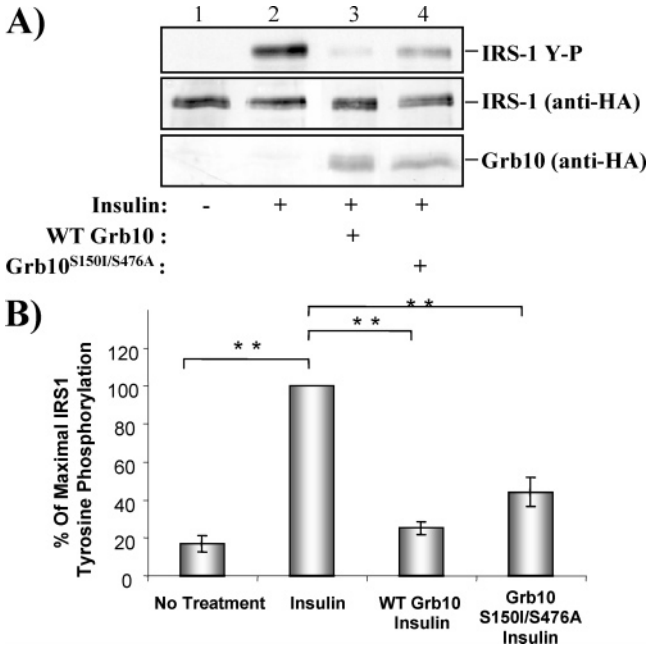

FIGURE 5: Phosphorylation of hGrb10ζ at Ser^{150} and Ser^{476} affects the ability of hGrb10ζ to inhibit insulin signaling. (A) CHO/IR cells transiently expressing either HA-tagged IRS-1 alone or IRS-1 plus wild-type or mutant hGrb10ζ were serum starved and treated with (+) or without (−) insulin (10^{-8} M) for 5 min. Tyrosine phosphorylation of IRS-1 in cell lysates was determined by Western blot using anti-phosphotyrosine antibody. The expression of IRS-1 and Grb10ζ was determined by Western blot using antibody to the HA tag and Grb10, respectively. (B) Quantitative analysis of the effect of either wild-type hGrb10 or $hGrb10\zeta^{S150I/S476A}$ on insulin-stimulated IRS-1 phosphorylation in cells. IRS-1 tyrosine phosphorylation was quantified using Scion Image and normalized to IRS-1 expression levels. The mean fold stimulation is expressed as a percentage of the maximum ± SE ($n = 3$). Key: *, $P < 0.05$; **, $P < 0.01$.

protein inhibited insulin-stimulated tyrosine phosphorylation of IRS-1 less efficiently than wild-type hGrb10 (Figure 5), suggesting that serine phosphorylation of Grb10 promotes Grb10-mediated inhibition of insulin signaling.

We found no difference between the ability of wild-type Grb10, $Grb10^{S150I}$, $Grb10^{S476A}$, $Grb10^{S150I/S476A}$, or $Grb10^{S150D/S476D}$ to associate with either MEK1 or Raf1, two kinases within the MAPK pathway that have been shown to interact with Grb10 (*30*) (data not shown). In addition to the IR and the IGF1R, Grb10 has also been found to interact with several cellular proteins including the ubiquitin ligase Nedd4 (*31*), which has been shown to recruit Nedd4 to the IGF1 receptor, resulting in IGF1 receptor degradation (*32*). Cotransfection experiments showed that introduction of either $Grb10^{S150I/S476A}$ or wild-type Grb10 resulted in similar levels of IGF1R degradation (data not shown), suggesting that phosphorylation of Grb10 at Ser^{150} and Ser^{476} does not play a role in the ability of Grb10 to mediate IGF1R degradation.

While our results show that serine phosphorylation of Grb10 at Ser^{150} and Ser^{476} is involved in the inhibitory role of Grb10 in insulin signaling, it is interesting to note that, in addition to these two sites, at least five additional major phosphopeptides exist in the phosphopeptide map of the in vivo labeled wild-type Grb10 (Figure 4). These findings suggest the presence of alternative phosphorylation sites and additional kinase(s) involved in the phosphorylation of Grb10 at these sites. It is possible that phosphorylation at the unidentified sites acts together with phosphorylation of Grb10 at Ser^{150} and Ser^{476} to regulate Grb10 function. Consistent with this, a very recent study proposed that Grb10 is phosphorylated at Ser^{428} and that phosphorylation at this site promotes the interaction between Grb10 and 14-3-3 (*24*). Identification of the remaining Grb10 phosphorylation sites and the kinases involved in the phosphorylation will provide important information in fully understanding how phosphorylation regulates the function of Grb10 in insulin signaling and action.

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