Grb10 has been proposed to inhibit or activate insulin signaling, depending on cellular context. We have investigated the mechanism by which full-length hGrb10γ inhibits signaling through the insulin receptor substrate (IRS) proteins. Overexpression of hGrb10γ in CHO/IR cells and in differentiated adipocytes significantly reduced insulin-stimulated tyrosine phosphorylation of IRS-1 and IRS-2. Inhibition occurred rapidly and was sustained for 60 min during insulin stimulation. In agreement with inhibited signaling through the IRS/PI 3-kinase pathway, we found hGrb10γ to both delay and reduce phosphorylation of Akt at Thr308 and Ser473 in response to insulin stimulation. Decreased phosphorylation of IRS-1/2 may arise from impaired catalytic activity of the receptor, since hGrb10γ directly associates with the IR kinase regulatory loop. However, yeast tri-hybrid studies indicated that full-length Grb10 blocks association between IRS proteins and IR, and that this requires the SH2 domain of Grb10. In cells, hGrb10γ inhibited insulin-stimulated IRS-1 tyrosine phosphorylation in a dose-dependent manner, but did not affect IR catalytic activity toward Tyr972 in the juxtamembrane region and Tyr1158/1162/1163 in the regulatory domain. We conclude that binding of hGrb10γ to IR decreases signaling through the IRS/PI 3-kinase/AKT pathway by physically blocking IRS access to IR.

The insulin receptor (IR) transmits signals through the actions of its intrinsic receptor tyrosine kinase. Ligand binding results in the autophosphorylation of the IR on multiple tyrosine residues (1, 2). Once phosphorylated, these residues serve as docking sites for specific intracellular effectors central to the transmission of the insulin signal. The insulin receptor substrate (IRS) family (IRS-1 through IRS-4) is one such group of proteins that recognizes the phosphorylated IR via their phosphotyrosine binding domains (PTB) (3, 4). The IRS-PTB domain associates with the NPXY motif surrounding tyrosine 972 in the juxtamembrane region of the IR, which promotes the IR-IRS interaction (5). Once in contact with the receptor, IRS is phosphorylated on multiple tyrosine residues by the IR tyrosine kinase. Numerous adaptor proteins and enzymes then associate with tyrosine-phosphorylated IRS via their Src homology 2 (SH2) domains and convey the insulin signal downstream (6, 7).

The insulin receptor-binding protein Grb10 is of considerable interest due to its potential to positively or negatively affect receptor-tyrosine kinase signaling. Grb10 is a member of a superfamily of adaptor proteins, which includes Grb7 and Grb14. This family of proteins shares several structural features, including a SH2 and a Pleckstrin homology (PH) domain (8). The C-terminal SH2 domain has been shown to associate with the IR (9, 10), and mutation of a critical arginine residue in this region of full-length Grb10 disrupts IR-Grb10 association in cells (11). A second domain termed the BPS (for between the Pleckstrin and SH2) has also been suggested as a second independent IR-interacting domain (9). Grb10 interacts with the regulatory kinase loop of the IR (10, 12, 13), although studies have also reported a Grb10-interacting region in the IR C terminus (14, 15). Several human Grb10 isoforms exist and most likely arise from alternative splicing. Two isoforms of hGrb10 differ in the PH domain. hGrb10γ encodes for the full-length protein, while Grb10α (previously named Grb-IR) lacks an intact PH domain as it contains a 46-amino acid deletion in this region (16, 17). A third isoform, hGrb10β, is identical to hGrb10γ with the exception of 58 amino acids at the extreme N terminus of the γ isoform (12, 17).

The functional role for Grb10 in insulin signaling remains controversial. Grb10 has been shown to positively stimulate insulin-induced mitogenesis (18). Microinjection of a peptide fragment including the BPS and SH2 domain of Grb10 inhibited insulin-stimulated DNA synthesis (13, 18), which, if functioning as a dominant negative, is consistent with Grb10 as a positive regulator for cell growth. Several studies have indicated an inhibitory role for Grb10 as well. Overexpression of hGrb10α negatively regulates insulin receptor-mediated tyrosine phosphorylation of GTPase-activated protein (GAP)-associated protein p60 and IRS-1 (16). Furthermore, binding of Grb10 via its BPS/SH2 domains has been shown to inhibit IR catalytic activity in vitro (19–21).

Stable expression of hGrb10α in CHO/IR reduces insulin-dependent phosphorylation of IRS-1 (16), although a direct effect of full-length Grb10 on IRS-1/2 and downstream effectors of the
Inhibition of Insulin Signaling by Grb10

In insulin signaling, such as Akt, has not been reported. However, several studies indicate that Grb10 may regulate downstream events in various signaling pathways. Overexpression of hGrb10y (Grb10y) in rat hepatocytes inhibits insulin-stimulated glycogen synthase activity, through a proposed novel pathway outside of the classical PI 3-kinase to Akt/glycogen synthase kinase-3 signaling (21). Grb10 has also been found to associate with tyrosine-phosphorylated c-kit receptor, and synergistically promote Akt activation. This study investigated the molecular mechanisms associated with tyrosine-phosphorylated IRS proteins. The anti-phosphotyrosine (RC-20) antibody was from Transduction Laboratories. IRS proteins were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and detected with specific antibodies. Quantitation of the proteins was performed by analyzing Western blots using the Scion Image program and normalized to the level of Akt expression in each experiment.

**Cell Lines and Reagents—**Chinese hamster ovary (CHO) cells have been previously described (16, 17). 3T3-L1 cells were from ATCC. Anti-HA antibody was obtained from BABCO. Monoclonal anti-Myc antibody was from Santa Cruz Biotechnology, Inc. Antibodies recognizing IRS-1 and IRS-2 were from Upstate Biotechnology. Akt and phospho-Akt (Thr308 and Ser473) antibodies were from New England Biolabs. The anti-phosphotyrosine (RC-20) antibody was from Transduction Laboratories. Phosphospecific antibodies to Tyr972, Tyr1158, and Tyr1160/1163 of the insulin receptor were obtained from BIOSOURCE International. Secondary antibodies conjugated to alkaline phosphatase and horseradish peroxidase were from Promega.

**Plasmids—**CDNAs encoding HA-tagged Grb10 and Myc-tagged Akt have been previously described (17, 24). HA-tagged Grb10, IRS-1, and IRS-2 were generated by single-stranded site-directed mutagenesis according to the protocol described by Kunkel et al. (25). The forward PCR primer 5'-GCTCTAGAGCTGCTGGTCTGCTG-3' and the reverse PCR primer 5'-CGAATTCTACAGACTGAGTGGTGG-3' were used to amplify the BSH2 fragment using pBex/Grb10 or pBex/Grb10R520G as templates. The reverse PCR primer 5'-GCGAATTCGTGCTGTGTCG-3' was used to amplify the SH2 domain fragment. PCR products were inserted in-frame with the HA tag using the XbaI and EcoRI restriction sites of the mammalian expression vector pBex16 (18). The forward PCR primer 5'-GCTCTAGATGCGGCCTCTGCTGGCCTC-3' and the reverse PCR primer 5'-CGAATTCTACAGACTGAGTGGTGG-3' were used to amplify the BSH2 fragment. The PCR products were inserted in-frame with the HA tag using the XbaI and EcoRI restriction sites of the mammalian expression vector pBex16. The forward PCR primer 5'-TGTCCTTAGAGCTGCTGGTCTG-3' and the reverse PCR primer 5'-GATCGCTGAGTCGAGTGGTGG-3' were used to amplify the SH2 domain fragment. PCR products were inserted in-frame with the HA tag using the XbaI and EcoRI restriction sites of the mammalian expression vector pBex16. The forward PCR primer 5'-GATCGCTGACCGACCCCATTCAGCTCCTC-3' and the reverse primer 5'-GCGAATTCACATTTCCGATCCGCGG-3' were used to amplify a 4.4-kb (SH2 domain) fragment from pRSKshnc-myc (a generous gift from Dr. Mark Greenspan). The individual PCR products were subcloned into an intermediate vector using the XhoI/SalI and SalI/EcoRI restriction sites. The resulting chimeric fragment was inserted in-frame with the HA tag using the XbaI and EcoRI restriction sites of pBex16. A plasmid encoding Myc-tagged IRS-1 was a generous gift from Dr. Mark Greenspan (27).

**MATERIALS AND METHODS**

**Yeast Transformation and Interaction Assay—**The yeast MATCHER LexA two-hybrid system reagents were purchased from Clontech. The yeast Saccharomyces cerevisiae strain GUS/EY44 (MATa, trp1, lys2, ade3, ura3, 6LexAOp-LEU2, LYS2) and the parental strain were transformed with pB42AD Grb10 constructs were generous gifts from Dr. Thomas A. Gustafson. GUSEY48 was sequencially transformed with plasmids constructs by the polyelectrolyte glycol/chitosan acetate method according to the Clontech protocol. Transformants were grown on SD-HisTrp agar plates for 3 days at 30°C. Four independent colonies were streaked onto SD-HisTrp agar plates, grown overnight, replicated onto SD plates containing galactose/raffinose/His-LeuTrp agar plates and incubated for 5 days at 30°C to induce expression of B42 fusion proteins and to determine interacting partners.

**Tri-Hybrid Disruption Assay—**GUSEY48 was sequentially transformed with pLexA-IR, pB42AD IRS-1 591aa and the reverse primer 5'-GATCGCTGACCGACCCCATTCAGCTCCTC-3' and the reverse primer 5'-GCGAATTCACATTTCCGATCCGCGG-3' were used to amplify a 4.4-kb (SH2 domain) fragment from pRSKshnc-myc (a generous gift from Dr. Mark Greenspan). The individual PCR products were subcloned into an intermediate vector using the XhoI/SalI and SalI/EcoRI restriction sites. The resulting chimeric fragment was inserted in-frame with the HA tag using the XbaI and EcoRI restriction sites of pBex16. A plasmid encoding Myc-tagged IRS-1 was a generous gift from Dr. Richard Roth (26). A plasmid encoding full-length human IRS-2 was a generous gift from Dr. Klein-Hitpass (27).

**RESULTS**

Grb10 inhibits Akt phosphorylation in response to insulin stimulation. In the insulin signaling cascade, elevated phospholipid levels result from PI 3-kinase activity and provide for the activation of downstream mediators, such as Akt (29, 30). To test the effects of hGrb10y on Akt activation, Myc-tagged Akt was expressed in CHO/IR cells together with HA-tagged

Grb10, hGrb10y, and hGrb10R520G were expressed in various CHO/IR cell lines. The effect of Grb10 on Akt phosphorylation was determined using the phosphotyrosine (RC-20) antibody. The results indicated that hGrb10y, hGrb10R520G, and hGrb10 increased Akt phosphorylation, as determined by the phosphotyrosine (RC-20) antibody.
hGrb10y. Insulin-stimulated Akt phosphorylation was detected using phosphospecific antibodies to Akt<sup>T308</sup> and Akt<sup>S473</sup>. Significant phosphorylation on both Akt<sup>T308</sup> and Akt<sup>S473</sup> was seen with a 5-min insulin stimulation (Fig. 1A, lanes 1 and 2, first and third panel), which is consistent with published results (31). The presence of hGrb10y greatly reduced phosphorylation on Akt<sup>T308</sup> (Fig. 1A, lanes 3 and 4, top panel). Phosphorylation at Akt<sup>S473</sup> was also decreased with hGrb10y overexpression (Fig. 1A, lanes 3 and 4, third panel). Quantitative analyses indicated that hGrb10y reduced Akt<sup>S473</sup> phosphorylation by ~50–60% and virtually abolished Akt<sup>T308</sup> phosphorylation (Fig. 1B).

Previous reports indicated that insulin rapidly induces phosphorylation of Akt<sup>T308</sup> and that this is stable during extended stimulation (31, 32). Furthermore, limited Akt<sup>S473</sup> phosphorylation in the basal state rapidly increases following growth factor stimulation (33). Since hGrb10y significantly inhibits Akt<sup>T308</sup> and partially inhibits insulin-stimulated Akt<sup>S473</sup> phosphorylation, we hypothesized that hGrb10y might have a temporal effect on Thr<sup>308</sup> and Ser<sup>473</sup> phosphorylation. Akt was overexpressed in CHO/IR cells in the absence or presence of hGrb10y. Cells were treated with 10 nM insulin for increasing times, and phosphorylation was detected in whole cell lysates using phosphospecific antibodies. hGrb10y delayed Akt phosphorylation at both sites, while concurrently decreasing the overall extent of phosphorylation for up to 1 h of insulin treatment (Fig. 1C).

**Full-length Grb10 Inhibits Insulin-stimulated Tyrosine Phosphorylation of IRS-1 and IRS-2**—The IRS proteins are rapidly phosphorylated in response to insulin stimulation. Since tyrosine-phosphorylated IRS-1/2 increases PI 3-kinase activity in the cell by binding the regulatory subunit of PI 3-kinase (7), we considered whether Grb10-mediated inhibition of Akt phosphorylation might arise from decreased signaling through IRS-1/2. A partial inhibition of insulin-stimulated tyrosine phosphorylation of IRS has been observed with hGrb10x (the isoform that lacks a PH-domain) (16), although the effects of full-length hGrb10y have not been investigated. hGrb10y and IRS-1 were co-expressed in CHO/IR cells and insulin-stimulated IRS-1 tyrosine phosphorylation was measured. In the absence of Grb10, IRS-1 was tyrosine-phosphorylated after 5 min of insulin stimulation (Fig. 2A, lanes 1 and 2). Insulin-stimulated tyrosine phosphorylation of IRS-1 was substantially inhibited when hGrb10y was co-expressed (Fig. 2A, lanes 3 and 4). IRS-1 tyrosine phosphorylation was inhibited in a dose-dependent manner (Fig. 2B, lane 2 versus lanes 3–6). We also examined the effects of hGrb10y on IRS-2 phosphorylation. Since little endogenous IRS-2 was detected in CHO/IR cells (data not shown), we co-expressed IRS-2 and hGrb10y in CHO/IR cells. We found that treatment of cells with insulin led to a significant increase in IRS-2 tyrosine phosphorylation (Fig. 2C, lane 2 versus lane 1). The insulin-stimulated IRS-2 tyrosine phosphorylation was greatly inhibited by overexpressing hGrb10y (Fig. 2C, lane 4 versus lane 2). These findings demonstrate that the Grb10-mediated inhibition is not specific for IRS-1.

hGrb10y Inhibits Endogenous IRS-1 and Akt Phosphorylation in Differentiated Adipocytes—To determine if hGrb10y inhibits phosphorylation of endogenous IRS and Akt in physiologically relevant cells, we used an adenovirus system to express hGrb10y in differentiated 3T3-L1 adipocytes. Infection efficiency was 85–90% as assessed by visualization of green fluorescence emitted from co-expressed GFP (data not shown). Insulin-stimulated phosphorylation of endogenous IRS-1 was inhibited (Fig. 3A), and Akt Thr<sup>308</sup> and Ser<sup>473</sup> phosphorylation significantly reduced in adipocytes expressing hGrb10y (Fig. 3B).

Grb10 Binding to IR Correlates with Inhibited IRS-1 Phosphorylation—Because hGrb10y delayed and sustained reduced levels of insulin-induced Akt phosphorylation (Fig. 1C), we investigated the temporal effects of hGrb10y on IRS-1 tyrosine phosphorylation. Time course studies indicated that hGrb10y inhibited IRS-1 tyrosine phosphorylation as early as 30 s after insulin stimulation, and that hGrb10y-mediated inhibition continued through an hour of insulin stimulation (Fig. 4A, top panel). Since hGrb10y inhibits insulin-stimulated IRS-1 tyro-
Inhibition of Insulin Signaling by Grb10

sine phosphorylation over an extended period, we studied the kinetics of hGrb10 binding to the IR. CHO/IR cells transiently expressing HA-tagged hGrb10 were stimulated with insulin for varying times, and hGrb10 was immunoprecipitated from the cell lysates with antibody to the HA tag. Bound IR was detected using an antibody specific to the subunit of IR.

hGrb10 rapidly associated with the IR within 30 s of insulin stimulation, and the association persisted through 60 min of hormonal treatment (Fig. 4B), which mirrors the speed and duration of hGrb10 inhibition of insulin stimulated IRS-1 tyrosine phosphorylation (Fig. 4A). We observed a decrease in Grb10 association following a 10-min insulin stimulation, which may result from subsequent dephosphorylation of the IR by activated tyrosine phosphatases.

Autophosphorylation of IR Is Not Affected by Grb10 Expression—IRS interacts directly with the tyrosine-phosphorylated NPEY motif in the IR juxtamembrane (4). Studies have indicated that autophosphorylation of the IR initiates at tyrosines 1158, 1162, and 1163 in the core catalytic domain (35), and phosphorylation in the C-terminal and juxtamembrane domains rapidly follows. Since Grb10 associates with the regulatory kinase domain of the IR (10, 12, 13), we considered that the effects of Grb10 on IRS tyrosine phosphorylation might arise from a decrease or loss in receptor autophosphorylation.

The insulin receptor was immunoprecipitated from cell lysates of either CHO/IR or CHO/IR stably expressing hGrb10 and the extent of insulin-stimulated phosphorylation at Tyr 972, for varying times, and hGrb10 was immunoprecipitated from the cell lysates with antibody to the HA tag. Bound IR was detected using an antibody specific to the subunit of IR. hGrb10 rapidly associated with the IR within 30 s of insulin stimulation, and the association persisted through 60 min of hormonal treatment (Fig. 4B), which mirrors the speed and duration of hGrb10 inhibition of insulin stimulated IRS-1 tyrosine phosphorylation (Fig. 4A). We observed a decrease in Grb10 association following a 10-min insulin stimulation, which may result from subsequent dephosphorylation of the IR by activated tyrosine phosphatases.

**Fig. 2.** Full-length Grb10 inhibits insulin-stimulated tyrosine phosphorylation of IRS. A, Grb10 inhibits insulin-stimulated tyrosine phosphorylation of overexpressed IRS-1 in CHO/IR cells. CHO/IR cells were transiently transfected with mammalian expression constructs encoding Myc-tagged IRS-1 and HA-tagged Grb10. Cells were serum-starved, stimulated with 10 nM insulin for 10 min, lysed, and IRS-1 immunoprecipitated with antibody to the Myc tag. Insulin-stimulated tyrosine phosphorylation of IRS-1 was detected by Western blot with a phosphotyrosine-specific antibody. Membranes were then stripped and reblotted for IRS-1 using an antibody to the Myc tag. Grb10 expression was detected in cell lysates using an antibody to the HA tag. B, Grb10 inhibits insulin-stimulated IRS tyrosine phosphorylation in cells in a dose-dependent manner. Myc-tagged IRS-1 was co-transfected with increasing amounts (1, 2, 4, or 8 μg) of HA-tagged Grb10 into CHO/IR cells. Cells were serum-starved, stimulated for 5 min with 10 nM insulin and lysed. Tyrosine phosphorylation of IRS-1 in cell lysates was determined as in A. C, full-length Grb10 inhibits IR-mediated tyrosine phosphorylation of IRS-2 overexpressed in CHO/IR cells. CHO/IR cells were transiently transfected with an expression construct encoding IRS-2, and either co-transfected with empty vector or an expression construct for Grb10. Twenty-four hours post-transfection cells were serum-starved and stimulated with 10 nM insulin for 10 min. Cell lysates were run on a 7.5% SDS-PAGE gel and tyrosine phosphorylation of overexpressed IRS proteins detected with a phosphotyrosine-specific antibody. Membranes were stripped and IRS-2 was detected with an antibody specific to the protein. The presence of Grb10 in the lysate was determined by probing with an antibody to the HA tag.

**Fig. 3.** Grb10 inhibits endogenous IRS-1 and Akt phosphorylation in differentiated adipocytes. A, Grb10 inhibits insulin-stimulated tyrosine phosphorylation of endogenous IRS-1. 3T3-L1 adipocytes were infected with either GFP or Grb10 adenovirus. Post-infection, cells were serum-starved and stimulated with 100 nM insulin for 5 min. Insulin-stimulated tyrosine phosphorylation of endogenous IRS-1 was detected in the lysates with a phosphotyrosine-specific antibody. Membranes were stripped and endogenous IRS-1 detected using an antibody specific to the protein. The amount of adenovirally expressed HA-tagged Grb10 was detected in the lysates using an antibody to the HA tag. B, Grb10 inhibits phosphorylation of endogenous Akt. 3T3-L1 adipocytes were treated and lysates prepared as in A. Insulin-stimulated phosphorylation of Akt at Thr308 and Ser473 was detected using phosphospecific antibodies. Endogenous Akt was detected using an antibody specific to the protein. Data is representative of three independent experiments.
were serum-starved and stimulated with 10 nM insulin for 5 min. The
serum-starved and stimulated with 10 nM insulin for the indicated
times prior to cell lysis. Lysates were separated by SDS-PAGE and
insulin-stimulated tyrosine phosphorylation detected using a phospho-
tyrosine-specific antibody. Blots were stripped and IRS-1 expression
determined by blotting with α-Myc antibody. Grb10y expression was
detected by Western blot with antibody to the HA tag. Data are representative of three
independent experiments.

Fig. 4. Grb10 binding to IR correlates with inhibited IRS-1 phosphorylation. A, IRS-1 tyrosine phosphorylation is rapidly and
stably inhibited by Grb10y. CHO/IR cells were co-transfected with Myc-tagged IRS-1 and empty vector or HA-tagged Grb10y. Cells were
serum-starved and stimulated with 10 nM insulin for the indicated
lanes 2 versus 4). While the presence of Grb10 has been shown to inhibit tyrosine phosphorylation of IR substrates in vitro (19–21), it is unclear whether this is
directly related to impaired catalytic activity. We considered
that if Grb10y does not significantly impair IR catalytic activity
in cells, then an alternative mechanism for Grb10-mediated
inhibition of substrate phosphorylation may exist. The results
in Fig. 5 illustrate that the catalytic activity of IR, at least
toward itself, is not significantly hindered by Grb10y overexpres-
sion in cells.

Grb10 Disrupts the Association of IRS-1/2 with the Insulin Receptor—To determine the mechanism behind Grb10-mediat-
ed inhibition of IRS-1/2 tyrosine phosphorylation, a yeast
tri-hybrid disruption assay was performed using IR as bait, IRS-1 or IRS-2 as prey and various Grb10 constructs (prey) as disrupters. The interaction between IR and IRS-1 (36) and
IRS-2 (37, 38) in yeast, as determined by growth on selective
media, has been well characterized. Co-expression of full-
length hGrb10β in the yeast tri-hybrid system prevented
growth on selective media (Fig. 6B), indicating that the N
terminus and PH domain are not required for inhibition of

Fig. 5. Insulin receptor autophosphorylation is unaffected by
Grb10y. CHO/IR cells alone or stably expressing Grb10y (HA-tagged)
were serum-starved and stimulated with 10 nM insulin for 5 min. The
IR was immunoprecipitated from the cell lysates, and samples were
separated by SDS-PAGE. IR tyrosine phosphorylation was detected
using phosphospecific antibodies to Tyr972, Tyr1158 and Tyr1162/1163.
Grb10y and IR expression levels were detected in whole cell lysates
using antibodies specific for the HA tag and the IRβ subunit,
respectively. Tyr1158, and Tyr1162/1163 was determined using phosphospecific antibodies. Phosphorylation at Tyr972 was not significantly
reduced in cells that stably expressed hGrb10y (Fig. 5, lanes 2
versus 4, first panel), suggesting that the reduction in IRS
tyrosine phosphorylation is not a direct consequence of an
impaired IRS docking site on the IR. Phosphorylation of the IR
on the three critical tyrosine residues in the regulatory loop
were similarly unaffected by expression of hGrb10y (Fig. 5,
lanes 2 versus 4, second and third panels). We considered
Grb10 Disrupts the Association of IRS-1/2 with the Insulin
Receptor—To determine the mechanism behind Grb10-mediated
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media, has been well characterized. Co-expression of full-
length hGrb10β in the yeast tri-hybrid system prevented
growth on selective media (Fig. 6B), indicating that the N
terminus and PH domain are not required for inhibition of

Fig. 6. Grb10 disrupts the interaction between IRS proteins
and the insulin receptor. A, yeast two-hybrid: interaction between
the insulin receptor (bait) and various Grb10 constructs (prey) was
determined by the ability to induce growth on selective media (+
). Grb10 constructs that do not interact with the insulin receptor do
not induce growth on selective media (−). B, Yeast tri-hybrid:
disruption of the insulin receptor (bait) and IRS-1 or IRS-2 (prey) interaction by
various Grb10 constructs (disruptants) was determined by the
inhibition of yeast growth on selective media.
IR/IRS-1/2 binding. To block SH2 domain function, the critical arginine residue at 462 was replaced with alanine (R462A) in the full-length and truncated (BPS/SH2) hGrb10β proteins. Both constructs interacted with IR (Fig. 6A and Ref. 9) and both also disrupted the IR/IRS-1/2 interactions similar to wild-type Grb10 (Fig. 6B). These data suggest that the BPS domain may be responsible for inhibiting IR/IRS-1/2 binding. However, the BPS (358–412) or SH2 (413–536) domains alone, which interact with IR in yeast two-hybrid experiments (Fig. 6A and Ref. 9), do not prevent yeast growth on selective media, indicating that the domains by themselves are unable to disrupt IR/IRS-1/2 interactions (Fig. 6B). Deleting the SH2 domain (Grb10ΔSH2 1–412 aa) or replacing it with the SH2 domain of Shc (p52) (Grb10ΔN–IGR-H9252−IR) does not inhibit association with the IR in yeast two hybrid experiments (Fig. 6A); however, removing or swapping the SH2 domain of Grb10 blocks the ability of Grb10 to inhibit the binding of IR/IRS-1/2 (Fig. 6B).

Furthermore, an inactive hGrb10β SH2 domain (413–536, R462A), which cannot associate with the IR in yeast two hybrid experiments (Fig. 6A and Ref. 9), can support interaction and disruption when combined with the BPS domain (Fig. 6A and B). Therefore, BPS directed disruption of IR/IRS-1/IRS-2 requires the structural presence of an active or inactive (R462A) Grb10 SH2 domain, which may physically block binding of IRS-1/2 to the NPXY972 motif.

Complete Inhibition of IRS Phosphorylation Requires an Intact Grb10 SH2 Domain—Results of the yeast tri-hybrid system indicated that disruption of IR/IRS interactions required both the BPS and SH2 domains. Furthermore, the Grb10 SH2 domain appeared to be essential and disruption occurred regardless of whether the SH2 domain was binding-competent. To determine if the BPS/SH2 region was necessary for inhibition of insulin-stimulated IRS phosphorylation in mammalian cells, we co-expressed full-length or BPS/SH2 fragments of wild-type or mutated forms of hGrb10 (12, 17). hGrb10 wild-type or mutated forms of hGrb10 cells, we co-expressed full-length or BPS/SH2 fragments of wild-type or mutated forms of hGrb10 (12, 17).

To determine if the BPS domain was necessary for inhibiting IR/IRS-1/2 binding, regardless of whether the SH2 domain was binding-competent. To block SH2 domain function, the critical arginine residue at 462 was replaced with alanine (R462A) in the full-length and truncated (BPS/SH2) hGrb10β proteins. Both constructs interacted with IR (Fig. 6A and Ref. 9) and both also disrupted the IR/IRS-1/2 interactions similar to wild-type Grb10 (Fig. 6B). These data suggest that the BPS domain may be responsible for inhibiting IR/IRS-1/2 binding. However, the BPS (358–412) or SH2 (413–536) domains alone, which interact with IR in yeast two-hybrid experiments (Fig. 6A and Ref. 9), do not prevent yeast growth on selective media, indicating that the domains by themselves are unable to disrupt IR/IRS-1/2 interactions (Fig. 6B). Deleting the SH2 domain (Grb10ΔSH2 1–412 aa) or replacing it with the SH2 domain of Shc (p52) (Grb10ΔN–IGR-H9252−IR) does not inhibit association with the IR in yeast two hybrid experiments (Fig. 6A); however, removing or swapping the SH2 domain of Grb10 blocks the ability of Grb10 to inhibit the binding of IR/IRS-1/2 (Fig. 6B).

Results of the yeast tri-hybrid system indicated that disruption of IR/IRS interactions required both the BPS and SH2 domains. Furthermore, the Grb10 SH2 domain appeared to be essential and disruption occurred regardless of whether the SH2 domain was binding-competent. To determine if the BPS/SH2 region was necessary for inhibition of insulin-stimulated IRS phosphorylation in mammalian cells, we co-expressed full-length or BPS/SH2 fragments of wild-type or mutated forms of hGrb10β along with IRS-1 in CHO/IR cells. The hGrb10β (yeast tri-hybrid studies) and hGrb10γ (mammalian cell studies) isoforms are identical outside of a 58-amino acid extension at the protein’s N terminus (12, 17). hGrb10β R462G corresponds to hGrb10γ R462G (Fig. 7A). As previously observed, insulin-stimulated IRS-1 phosphorylation was inhibited in the presence of full-length wild-type hGrb10γ (Fig. 7B). Consistent with our yeast tri-hybrid findings, co-expression of the BPS/SH2 fragment in cells similarly decreased insulin-stimulated IRS-1 phosphorylation (Fig. 7B, first panel, lane 4). Inhibition of IRS-1 tyrosine phosphorylation correlated with Grb10 association with the IR, and the BPS/SH2 fragment was sufficient to maintain this interaction (Fig. 7B, third panel, lanes 3 and 4). Interestingly, IRS-1 tyrosine phosphorylation was partially restored with either the BPS/SH2 R520G fragment or full-length Grb10γ R520G (Fig. 7B, first panel, lanes 5 and 8). These findings suggest that an active SH2 domain is necessary for complete inhibition of IRS tyrosine phosphorylation in cells. Studies have indicated that the BPS and SH2 domains may act cooperatively to facilitate the interaction of Grb10 with the IR (9). Full-length Grb10γ with an inactive SH2 domain is unable to co-immunoprecipitate detectable levels of the IR (Fig. 7B, lane 8, third panel and Ref. 11). Similarly, the BPS/SH2 R520G fragment was unable to co-immunoprecipitate detectable amounts of the IR (Fig. 7B, lane 5, third panel). Our results suggest that the BPS domain is necessary, but not sufficient to mediate a tight association with the IR in cells. In the absence of an intact SH2 domain, the interaction between Grb10 and the IR may be transient and therefore undetectable by co-immunoprecipitation (Fig. 7B, third panel, lanes 5 and 8 versus 3 and 4). However, this transient association is sufficient to affect IR/IRS-1 interactions as indicated by the partial inhibition of IRS-1 tyrosine phosphorylation (Fig. 7B). To confirm the necessity of the SH2 domain for Grb10-IR association and its effect on the inhibition of insulin-stimulated IRS tyrosine, we tested two additional Grb10γ constructs. By either replacing the Grb10 SH2 domain with the SH2 domain of Shc (p52) or by deleting this domain altogether, IRS-1 tyrosine phosphorylation was restored, 41 and 30%, respectively (Fig. 7B, first panel, lanes 6 and 7). Immunoprecipitation with either the Grb10γ 1–491:ShcSH2 chimer or Grb10γ ΔSH2 revealed that the BPS domain alone cannot maintain a sufficient interaction to co-immunoprecipitate the IR (Fig. 7B, lanes 6 and 7, third panel). Taken together with the yeast tri-hybrid results, the SH2 domain makes dual contributions to Grb10-mediated inhibition of IRS phosphorylation: in cells, the SH2 domain stabilizes the interaction of Grb10 with the IR, and with a stable Grb10/IR interaction, the SH2 domain sterically hinders IRS access to its binding motif on the IR.

**DISCUSSION**

Grb10 has been shown to play both a positive and negative role in insulin signaling, and these differences appear to depend both on cell type and on the Grb10 splice variant being...
Inhibition of Insulin Signaling by Grb10

studied. To understand these functional differences, we investigated the molecular mechanism of Grb10 action at the insulin receptor. Here, we show that full-length human Grb10 has an inhibitory effect on insulin-stimulated signaling through the IRS/PI 3-kinase pathway to Akt. Using a yeast tri-hybrid system, we found Grb10 can disrupt the association of IRS proteins with the IR. The Grb10 SH2 domain is essential for this disruption and for complete inhibition of IRS tyrosine phosphorylation in cells. Our findings support a mechanism for Grb10-mediated inhibition of IRS/PI 3-kinase/Akt signaling, in which association of IRS proteins with the IR kinase domain and/or the juxtamembrane NPXY motif (Tyr772) is sterically hindered by Grb10.

Previous studies have concluded that blocking IR catalytic activity by either full-length Grb10 or domain fragments results in decreased substrate phosphorylation (19, 20). In agreement with these studies, we have observed a Grb10-mediated decrease in phosphorylation of IR substrates, including IRS-1/2 (this study) (16), p62(dsh) (16, 39), and Shc (data not shown). However, Mournier et al. (21) reported that overexpression of hGrb10 did not affect IRS tyrosine phosphorylation, PI 3-kinase activity or Akt activity in rat hepatocytes, although IR kinase activity and IR autophosphorylation were reportedly reduced. Our results in CHO/IR cells show that the catalytic activity of the RTK toward itself is not significantly impaired by Grb10. To a degree this is intuitive since tyrosine phosphorylation of the IR is necessary for Grb10 association. The rapid association of Grb10 with the IR could suggest that if Grb10 blocked the enzymatic activity of the RTK, autophosphorylation of the IR would be reduced in CHO/IR/H9253. However, we found that the overall tyrosine phosphorylation of the IR was also unaffected by the presence of hGrb10 (data not shown). It is possible that the effects of Grb10 on catalytic activity arise from conformational changes that sterically hinder access of the kinase with its substrates. While we can not exclude inhibition of enzymatic activity of the insulin receptor for IRS-1/2 as a cause for decreased substrate phosphorylation, the observation that hGrb10 physically disrupts the interaction between IR and IRS proteins using the yeast tri-hybrid system supports structural interference as a mechanism for Grb10-mediated inhibition. The interaction of IRS proteins with the IR is transient in nature, and several negative feedback mechanisms exist in cells to prompt rapid dissociation of tyrosine-phosphorylated IRS proteins with the receptor (40–42). As a result, we were unable to detect an interaction between IR and IRS proteins by co-immunoprecipitation (data not shown), and cannot completely assess physical disruption as an alternative mechanism for Grb10-mediated inhibition in mammalian cells.

Although both the BPS and SH2 domains can independently interact with the IR, the two regions together are necessary for disruption of interactions between IRS-1/2 and IR. Our results with the yeast tri-hybrid system indicate that the BPS domain with either an active or inactive SH2 domain is sufficient for disruption. This would suggest that the BPS domain mediates the interaction with the IR and the SH2 domain facilitates a physical disruption of IR/IRS interaction. When the Grb10 SH2 domain is deleted or replaced with the Shc SH2 domain, the IR/IRS interaction is not disrupted although both constructs interact with the IR in yeast two-hybrid assay. These findings support a structural role for the Grb10 SH2 domain in hindering IR/IRS interactions. However, Grb10 molecules lacking an intact SH2 domain cannot support complete inhibition of IRS tyrosine phosphorylation in mammalian cells (Fig. 7B). Interestingly, while the BPS domain with either an active or inactive SH2 domain disrupted IR/IRS interactions in the yeast tri-hybrid studies, only Grb10 (full-length and BPS/SH2 fragment) with an active SH2 domain inhibited insulin-stimulated IRS-1 tyrosine phosphorylation in mammalian cells. Complete inhibition appeared to correlate with the ability of Grb10 to co-immunoprecipitate the IR. In contrast with results using yeast two-hybrid, in which the BPS domain is sufficient for interaction with the IR, regardless of an active SH2 domain (Ref. 9 and Fig. 6), only Grb10 with an active SH2 domain could co-immunoprecipitate the IR in cells. The high sensitivity of the yeast system may allow for the detection of the BPS-mediated IR interaction and may override the need for an active SH2 domain. In mammalian cells, the SH2 domain may need to facilitate or strengthen BPS-mediated association with the IR. For this reason, we could not fully assess the structural requirement of the SH2 domain in physically blocking IRS-1/2 access to the IR in cells. In addition, we were unable to express fragments encoding only the BPS or SH2 domains, most likely due to their instability in mammalian cells. While we were unable to detect Grb10 association with the IR when the SH2 was inactivated or deleted, partial inhibition of IRS tyrosine phosphorylation with overexpression of these mutants suggests that some interaction via the BPS domain is likely.

Full-length hGrb10a significantly reduced phosphorylation of Akt on both Thr308 and Ser473 in CHO/IR cells. A similar inhibitory effect on phosphorylation of endogenous Akt was seen with adenosival expression of hGrb10a in differentiated adipocytes. Our findings differ from a recent study reporting mGrb10 functioned as a positive regulator of Akt in the c-kit signaling pathway by promoting relocalization to the membrane (22). We do not find hGrb10 to co-immunoprecipitate with Akt in CHO/IR cells (data not shown). Protein interactions with Grb10 may be species-specific. Receptor-associated variations in recruited signaling pathways may also account for the observed differences in Grb10 function. IRS signaling is directly impaired by Grb10 interactions with IR and inhibition of Akt activity by hGrb10a may stem from a specific requirement for IRS proteins and their supporting role in the activation of PI 3-kinase in response to insulin stimulation.

In this study, the mechanism for Grb10-mediated inhibition of insulin signaling was investigated. We found that full-length hGrb10 could significantly inhibit insulin-stimulated tyrosine phosphorylation of IRS-1 and IRS-2 and subsequently delay signaling to the downstream effector, Akt. Autophosphorylation of the IR is not impaired by the binding of Grb10 to the receptor, which indicates functional activity of the RTK in cells. hGrb10a inhibits insulin-stimulated tyrosine phosphorylation of IRS in a dose-dependent manner, and the interaction between IRS and the IR is disrupted when full-length Grb10 is used in the yeast tri-hybrid system. These studies describe a new molecular mechanism for Grb10-mediated inhibition. By structurally hindering access to the IR kinase domain and/or the NPXY motif (Tyr772), Grb10 inhibits tyrosine phosphorylation of IRS-1 and IRS-2 and functions to decrease intracellular insulin signaling through the IRS/PI 3-kinase pathway.

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Grb10 Inhibits Insulin-stimulated Insulin Receptor Substrate (IRS)-Phosphatidylinositol 3-Kinase/Akt Signaling Pathway by Disrupting the Association of IRS-1/IRS-2 with the Insulin Receptor

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