Human GRB-IRβ/GRB10

SPLICE VARIANTS OF AN INSULIN AND GROWTH FACTOR RECEPTOR-BINDING PROTEIN WITH PH AND SH2 DOMAINS*

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cDNA clones encoding human (h) Grb7 and a previously unknown protein with high homology to hGrb-IR and mGrb10 (where m indicates mouse) were found by screening expressed sequence tag data bases. hGrb7 mRNA expression is greatest in pancreas and restricted to a few other tissues. The second protein termed hGrb-IRβ/Grb10 contains an intact PH domain and lacks the 80-residue mGrb10 insertion. Expression is greatest in pancreas and muscle but occurs in nearly all tissues. hGrb-IRβ/Grb10 and hGrb-IR likely arise as alternative mRNA splicing products of a common gene. Reverse transcriptase-coupled polymerase chain reaction shows both mRNAs in muscle. In cells, Grb-IRβ/Grb10 protein translocates from cytosol to membrane upon insulin stimulation, most likely due to direct interactions with the insulin receptor. These interactions are mediated by the SH2 domain and additional regions of the protein. Studies with mutated receptors and synthetic phosphotyrosines show that the hGrb-IRβ/Grb10 SH2 domain binds at least two sites in the insulin receptor: the kinase activation loop > the juxtamembrane site. hGrb-IRβ/Grb10 also binds a 135-kDa phosphoprotein in unstimulated 3T3-L1 adipocytes; binding is reduced upon insulin stimulation. In addition, the c-Abl SH3 domain binds Grb-IR/Grb10, whereas Fyn, phosphatidylinositol 3-kinase p85, and Grb2 SH3 domains do not. The site of c-Abl SH3 domain interaction is highly conserved within the Grb-IR/Grb10/Grb7/Grb14 family. hGrb-IRβ/Grb10 also binds platelet-derived growth factor and epidermal growth factor receptors, suggesting a broader role in the signaling pathways of numerous receptors. We conclude that hGrb-IRβ/Grb10 is a widely expressed, PH and SH2 domain-containing, SH3 domain-binding protein that functions downstream from activated insulin and growth factor receptors.

Many of the effects of activated tyrosine kinase-linked receptors are mediated by cascades of intracellular tyrosine phosphorylation reactions. Receptors with intrinsic kinase activity typically phosphorylate themselves, and in many cases the phosphorylated receptor tyrosines serve as docking sites for SH2 domain proteins (1, 2). Since many SH2 domain proteins either are enzymes or associate with enzymes, these interactions provide a mechanism for recruiting catalytic effectors to the activated receptor. In the case of insulin signaling, auto-phosphorylation activates the receptor kinase (3, 4) and creates a docking site for substrate protein PTB domains (5, 6). Both effects are necessary to trigger intracellular pathways via the substrates IRS-1 and Sbe. However, the SH2 domain effectors of insulin action bind primarily to the phosphorylated substrate proteins rather than the insulin receptor itself.

The recent discovery of an SH2 domain protein called Grb-IR was met with considerable interest because it binds the insulin receptor and not its substrates (7). However, this Grb7-like protein reportedly inhibits insulin signaling and contains an unusual 46-residue deletion within its apparent PH domain. Therefore, we have considered the possibility that additional related proteins (potentially with intact domains) might exist to provide positive signals downstream from the insulin receptor. Three members of the Grb7 family (Grb7, Grb10, and Grb14) have been identified by screening cDNA expression libraries with phosphorylated fragments of the EGF receptor (8–10). Although each is derived from a distinct genetic locus, they share a common domain architecture: a C-terminal SH2 domain and >300 residues of extended homology that encompasses a PH domain. mGrb10 also contains an 80-residue insertion, relative to mGrb7 and hGrb14. Its function is unknown. Although the presence of SH2 and PH domains strongly implies a role for these proteins in cellular signaling, their physiologic functions remain vague. Nevertheless, all have been implicated in neoplastic conditions. mGrb7 binds and is coamplified with HER2/neu in certain types of breast cancer (11). mGrb10 binds the Ret receptor (12), whose gene (the ret protooncogene) is rearranged and activated in certain thyroid carcinomas and contains germ line mutations associated with syndromes of multiple endocrine neoplasias (13, 14). And expression of Grb14 may be elevated in estrogen receptor-positive breast cancer cell lines and certain prostate cancer cells (10). hGrb-IR and mGrb10 proteins share regions of high homology, although the two are not simple species variants. hGrb-IR has not been implicated in oncogenesis.

Expressed sequence tag data bases (dbEST) were screened as a strategy to identify related proteins. Two clones were found. One encodes human Grb7. Further sequencing of the second clone revealed a previously unknown protein with high homology, although the presence of SH2 and PH domains strongly implies a role for these proteins in cellular signaling, their physiologic functions remain vague. Nevertheless, all have been implicated in neoplastic conditions. mGrb7 binds and is coamplified with HER2/neu in certain types of breast cancer (11). mGrb10 binds the Ret receptor (12), whose gene (the ret protooncogene) is rearranged and activated in certain thyroid carcinomas and contains germ line mutations associated with syndromes of multiple endocrine neoplasias (13, 14). And expression of Grb14 may be elevated in estrogen receptor-positive breast cancer cell lines and certain prostate cancer cells (10). hGrb-IR and mGrb10 proteins share regions of high homology, although the two are not simple species variants. hGrb-IR has not been implicated in oncogenesis.

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hGrb-IR and mGrb10, which we refer to as hGrb-IR/Grb10. It has an intact PH domain and lacks the mGrb10 insertion. hGrb-IR/Grb10 and hGrb-IR probably represent alternative mRNA splicing products of a common gene. Both mRNAs are expressed in muscle, a major site of insulin action. hGrb-IR/Grb10 protein is present in the cytosol of unstimulated Rat1 fibroblasts and translocates to the membrane following insulin stimulation. We have characterized hGrb-IR/Grb10 interactions with activated insulin, EGF, and PDGF receptors and SH3 domain proteins. We conclude that hGrb-IR/Grb10 is a previously unknown signaling protein that may function downstream from activated insulin and growth factor receptors.

MATERIALS AND METHODS

**DNA Sequencing**—Automated DNA sequencing was carried out at the Joslin Diabetes Center DNA Core Facility with an Applied Biosystems Model 373 DNA sequencer. Sequencing substrates were produced by unidirectional nested deletions of plasmid substrates, with ExoIII/S1 treatment. Sequence assembly and analysis was carried out with Genetec Computer Group Wisconsin Package version 8.1 software.

**RT-PCR**—Candidate hGrb10 5′-RACE products were amplified by PCR from a gt11 human skeletal muscle cDNA library (Clontech). Each 100-μl reaction contained 100 ng of phage DNA, 1.5 mM MgCl₂, 0.5 units Tag DNA polymerase (Perkin-Elmer), and 50 μM each of a lambda upper primer (U3: 5′-GATTTTGCCGAGCAGTTGCA-3′) and a lower primer (7-2: 5′-CCCGTGAAACCAGTGCTGTG-3′) which anneals to cDNA plasmid clone HCEEI20 (see “Results” and “Discussion”). Thirty PCR cycles were conducted as follows: 94°C for 20 s, a variable temperature for 30 s, and 72°C for 2 min. The variable temperature was decreased in increments of 0.5°C from 70 to 55°C. Three predominant PCR products of 0.4, 1.0, and 1.7 kb were purified, cloned in pBluescript II SK (Stratagene), and sequenced. A full-length cDNA was obtained by combining the 1.7-kb 5′-RACE product and HCEEI20 using PCR-mediated fusion, with primers U3 and 7-1 (5′-TGGAGGGACCTT-GTGCTAC3′) and T7 and 7-1R (5′-GGTACCGAATTCCTCCGAC-3′), respectively.

**RT-PCR**—Analytical polymerase chain reactions were carried out with cDNA prepared from human skeletal muscle poly(A) RNA (Clontech), using reaction conditions described for 5′-RACE. Two upper (A1: 5′-GTGAGCTGACCCTGCTGGAG-3′, nucleotide position 56, hGrb-IR/Grb10 cDNA; A2: 5′AGACCTAAGCTTGGTGCTC3′, position 141, hGrb-IR/Grb10 cDNA) and two lower (L1: 5′GGTACCGAATTCCTCCGAC-3′, position 1075, hGrb-IR/Grb10 cDNA; L2: 5′GGGTATCCACAGTGGCTG-3′, position 1582, hGrb-IR/Grb10 cDNA) primers were used to identify hGrb-IR/Grb10 transcripts. Two additional primers were used to identify Grb-IR transcripts (B1: 5′-GAAAGAGCGAGAGAAGGACCC3′, position 11, hGrb-IR/Grb10 cDNA; accesson U34355); and B1: 5′-ACCTGCTGACCTGCTG-3′, position 324, hGrb-IR/Grb10 cDNA) primers were used in paired combinations: A1/P1, A2/P1, B1/P1, A2/P2, and A2/7–2.

**Northern Blot Analyses**—The inserts of the plasmid cDNA clones HUKCW90 and HCEEI20 were amplified by PCR with the T3 and T7 primers. Purified PCR fragments, labeled with [32P]dATP to greater than 2 × 10⁶ cpm/μg by the random hexamer method, were hybridized to human multiple tissue Northern blots (Clontech). The membranes were washed at high stringency and exposed to storage phosphor screens (Molecular Dynamics). Northern blot manipulations were carried out according to procedures recommended by the manufacturer.

**hGrb-IR/Grb10 Fusion Proteins—**EcoRI and Xhol sites were introduced by PCR immediately upstream and downstream of the regions of the hGrb-IR/Grb10 cDNA encoding residues 1–356 and 455–536. The DNA fragments were subcloned into the corresponding sites in a pGEX4T-3 (Pharmacia Biotech Inc.) plasmid and used to transform Escherichia coli strain LE392 and XL-1 Blue (Stratagene). Following induction of protein expression with isopropylthio-β-d-galactoside and cell collection and lysis, the protein was purified by affinity chromatography using an immobilized glutathione-agarose column (Molecular Probes). GST (glutathione S-transferase) fusion proteins were eluted with 20 mM glutathione and dialyzed 24 h with 100 mM ammonium bicarbonate containing 1.0 mM diethiothreitol. The proteins were concentrated using a Centricon-10 device (Amicon).

**Cell Lines—**3T3-L1 cells were grown in DMEM containing 10% calf serum. To induce the differentiation of 3T3-L1 fibroblasts into adipocytes, cells in DMEM containing 10% fetal bovine serum were treated for 3 days with 0.5 mM 1-methyl-5-isoxybutyamine, 0.4 μg/ml dexamethasone, and 5.0 μg/ml insulin. Cells were then maintained for an additional 15–15 days in DMEM containing 10% fetal bovine serum and 5.0 μg/ml insulin. Prior to experiments, the 3T3-L1 adipocytes were serum-deprived for 48 h in DMEM containing 0.2% bovine serum albumin. Rat1 fibroblasts overexpressing the wild-type human insulin receptor (HIR) were kindly provided by J. White, University of California, San Diego. Mutated human insulin receptors (15–17) were obtained by solubilizing transfected Chinese hamster ovary cells as described (18) (the cells were generously provided by C. R. Kahn and M. White, Joslin Diabetes Center).

**Precipitations with Antibodies and Fusion Proteins and Western Blotting**—Adipocyte lysates were solubilized in 1% Triton X-100 for 10 min at 4°C and incubated with either GST-fusion protein (4 μg) bound to glutathione-agarose beads (Molecular Probes) or antibodies bound to protein A-Sepharose (Pharmacia). Proteins eluted from the washed pellets were separated by SDS-PAGE and transferred to a poly(vinylidene difluoride) membrane (Immobilon PVDF, Millipore) by electroblotting. Membranes were incubated either with GST-fusion protein (4 μg/ml leupeptin, pH 7.4, 0.1% Tween 20) and 2% gelatin for 2 h at 22°C and reacted with specific antibodies in saline buffer containing 5% bovine serum albumin for 16 h at 4°C. Proteins were identified following incubation with horseradish peroxidase-linked second antibody using an enhanced chemiluminescence method, as instructed (Pierce). In indicated experiments, immunoblots were stripped with 2% SDS and 100 mM β-mercaptoethanol in 62.5 mM Tris-HCl, pH 6.7, for 30 min at 50°C and re-blotted. The anti-Grb10 antibody (against residues 190–621 of mGrb10) was provided by B. Margolis (University of Michigan), anti-Tyr(P) (4G10) antibodies were from UBL, and the anti-insulin receptor antibody was provided by B. Cheatham (Joslin Diabetes Center).

**Cellular Fractionation—**HIRc fibroblasts were serum-deprived for 16 h prior to stimulation with 10⁻⁴ μM insulin for 5 min at 37°C. The cells were collected by scraping in ice-cold buffer A (50 mM HEPES, 150 mM NaCl, 10 mM EDTA, 10 mM NaP₂O₄, 100 mM NaF, 2 mM sodium vanadate, 0.5 mM phenylmethanesulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, pH 7.4) and further disrupted by multiple passages through a 26-gauge needle. After a slow speed centrifugation (600 × g for 5 min) to remove nuclei and cellular debris, the mixtures were centrifuged for 1 h at 10⁵ × g and the supernatant (S₁) was considered to be cytosolic fractions. The pellets were resuspended in buffer A containing 1% Triton X-100 and mixed for 45 min at 4°C. These solutions were centrifuged 1 h at 10⁵ × g and 4°C. Supernatant solutions are considered to be the membrane fraction and pellets are Triton-insoluble fractions. Proteins were separated by SDS-PAGE and identified by immunoblotting with specific antibodies.

**Peptide Synthesis**—Cleavage of Phosphopeptides corresponding to phosphorylation sites within the insulin receptor were synthesized manually using Fmoc-protected amino acids and O-benzotriazolyl-N,N,N’,N’-tetramethylethuronium hexafluorophosphate as the coupling reagent: the monophosphoryl juxtamembrane site, pYN90 (SSNpEPLY SASDVE-NH₂), the bisphosphoryl C terminus, pY2CT (GFKRSpYEE-HIPyTMYNG-NH₂), and the triphosphoryl activation loop, pYNLoop (MTRDpYEYTDpYpYRRGGKGG-NH₂) (19). Peptides were purified by preparative high performance liquid chromatography and characterized by analytical high performance liquid chromatography and electrospray mass spectrometry. Solubilized, wheat germ agglutinin-agarose-purified insulin receptors (19, 20) were reacted with 10⁻⁴ μM insulin for 45 min and phosphorylated in the presence of 15 μM ATP, 8 mM MgCl₂, and 4 μM MnCl₂ for 30 min, all at 22°C. Immobilized hGrb-IR/ Grb10 GST-S52 domain or GST full-length protein (2 μg) was incubated at 22°C for 30 min with the phosphorylated insulin receptor. Incubations were conducted in the presence and absence of the phosphopeptides, and bound insulin receptor was identified by immunoblotting.

**SH3 Domain Binding—**Human Fyn SH3 domain (residues 84–148) (provided by H. Band, Brigham and Women’s Hospital) (21), murine C-Abl SH3 domain (residues 84–145) (provided by G. White, Children’s Hospital, Boston), and PI-3 kinase p85 SH3 domain (residues 1–80) (provided by L. Cantley, Beth Israel Hospital) (22) were expressed as GST fusion proteins using pGEX-2T vectors (Pharmacia), as described. Full-length murine Grb2 (provided by M. Moran, University of Toronto) was expressed as a GST fusion protein using pGEX-3X vector (Pharmacia). Methods for fusion protein expression and purifi-
RATION were as described above. HIRc fibroblasts were solubilized, and clarified cell lysates were incubated with 2 \( \mu \)g of immobilized SH3 domain or Grb2 for 1 h at 4 °C. Incubations were conducted in the presence or absence of 3BS peptide (SLPAIPNPFPEL). Pellets were washed, proteins were separated by SDS-PAGE, and Grb-IR/Grb10 isoforms were identified by immunoblotting.

RESULTS

Identification of Human cDNAs Encoding Grb7 and Grb-IR/Grb10 Related Proteins—Genome sequencing initiatives have generated many millions of nucleotides of human DNA sequence. Much of this information was derived from expressed sequence tags (23). Although expressed sequence tags typically represent incomplete gene sequences, partial protein coding sequences can be deduced from the data, and in some cases it is possible to predict the function of the encoded protein based on sequence homology. This is particularly true for proteins with relatively short but characteristic homology domains. Expressed sequence tags 370184 and 201358 (TIGR HCD Accession D70184 and C01358) were identified by the BLASTX homology search program as potential SH2 domain proteins. Further inspection and alignment with all known SH2 domains suggested that these proteins might be members of the Grb7/Grb10 family of SH2 domain proteins, as these are the only known proteins with C-terminal SH2 domains that end with the residues Val-Ala-Leu (e.g. Fig. 1). The corresponding plasmid cDNA clones (HUKCW90 and HCEEI20) were obtained from Damien Dunnington (SmithKline Beecham Pharmaceuticals), and the nucleotide sequences of the inserts were refined and extended. HUKCW90 was derived from a human uterine cancer cDNA library. The insert contains a 2.0-kb cDNA encoding an intact protein identical to human Grb7. The sequence of hGrb7 has not been published in journal format but is available (GenBank D43772). HCEEI20 was derived from a human cerebellum cDNA library. It contains a 3.5-kb cDNA encoding a protein fragment whose C-terminal sequence (residues 388–548) is identical to human Grb-IR (7) and very similar to murine Grb10 (9).

Isolation and Sequence Characterization of the Full-length cDNA—A lig11-specific primer and an HCEEI20-specific primer were used to amplify cDNAs carrying 5’ and 3’ extensions of HCEEI20 from a human skeletal muscle cDNA library by PCR (see “Tissue Distribution,” below). Three 5’-RACE (rapid amplification of cDNA ends) candidates were isolated, subcloned, and sequenced. The entire sequences of the 1.0- and 0.4-kb products were found within the 1.7-kb product. The combination of sequences of HCEEI20 and the 1.7-kb RACE product yielded an open reading frame which potentially encodes a 536-amino acid protein (Fig. 1). The putative initiation site is preceded in-frame by two termination codons. The termination codon after residue 536 is followed by a series of additional termination codons (data not shown; cDNA sequence deposited, GenBank accession number U69276). Residues 233–355 fit the consensus for an intact PH domain (24, 25).
Thymus and peripheral blood leukocytes showed very low or no detectable hGrb-IR/Grb10 mRNA. The transcript in brain is shifted slightly upwards, perhaps indicating a variant mRNA. Two additional 4.8- and 3.1-kb transcripts were detected in skeletal muscle, suggesting alternative polyadenylation sites or differential splicing. The reported tissue distribution for hGrb-IR mRNA should be identical because the probes in the studies were derived from common regions of the two cDNAs (7). hGrb-IR was detected primarily in skeletal muscle and pancreas, and there were three transcripts in skeletal muscle (reportedly 6.5, 5.0, and 2.2 kb). The relative abundance of the message in alternative tissues is less clear. The mGrb10 expression pattern is distinct. A single 6.0-kb transcript is predominant in heart and kidney, with lesser amounts detected in brain and lung (9).

Presence of Two Distinct Transcripts Confirmed by RT-PCR—The coexistence of distinct mRNAs for hGrb-IR and hGrb-IR/Grb10 was confirmed by reverse transcriptase-coupled PCR (RT-PCR) on polyadenylated mRNA derived from human skeletal muscle (Fig. 3). Two upper primers (A1 and A2) specific for Grb-IR/Grb10, and a lower primer (P1) annealing to a region common to both Grb-IR/Grb10 and Grb-IR, gave the predicted PCR fragments of 288 and 203 bp, respectively (see Fig. 1B for a projection of primer binding sites on Grb-IR/Grb10 and Grb-IR). This result independently confirms the presence of an mRNA corresponding to 5′-untranslated and coding sequences for Grb-IR/Grb10. In parallel, an upper primer (B1) specific for Grb-IR and common primer P1 gave the predicted PCR fragment of 231 bp. Therefore, mRNAs with elements unique to Grb-IR and Grb-IR/Grb10 coexist in human skeletal muscle. The yield of Grb-IR products was significantly lower than that of the Grb-IR/Grb10 products, consistent with the possibility that the Grb-IR/Grb10 transcript is more stable or that Grb-IR/Grb10 is expressed more abundantly. A third isoform of the Grb10/Grb-IR family in muscle is indicated by the presence of an additional 450-bp PCR product in the Grb-IR reaction. The primers used for its identification (B1 and P1) suggest that this isoform could have a third N-terminal sequence.

Additional experiments confirmed the domain architecture of hGrb-IR/Grb10 and validated our sequencing strategy (Fig. 3). A second lower primer (P2) was designed to anneal mRNA encoding the segment of hGrb-IR/Grb10 PH domain that is lacking in hGrb-IR. When used in combination with upper...
Grb10 Moves from Cytosol to Membrane Fractions Upon Insulin Stimulation—Many signaling events occur at discrete locations in cells, and modular elements of protein structure domains (e.g. SH3, PH, and SH2 domains) frequently participate in subcellular compartmentalization (1, 2, 26). HIRc fibroblasts that overexpress human insulin receptors were fractionated to determine the cellular location of mGrb-IR/Grb10 isoforms. In unstimulated HIRc cells, the protein is in the cytosol (Fig. 4). However, upon insulin stimulation a remarkable amount of mGrb-IR/Grb10 redistributes to the membrane fraction. This may be through direct binding to the insulin receptor or interactions with additional cellular constituents. While the polyclonal anti-Grb10 antibody (from B. Margolis) recognizes three predominant species, only intact p65 mGrb10/Grb-IR redistributes upon insulin activation. Furthermore, only p65 binds SH3 domains (see below), suggesting that the lower molecular weight immunoreactive species are functionally (and perhaps structurally) unrelated. Control studies show that insulin receptors present in cell membranes do not redistribute. Under more physiologic conditions where fewer receptors are present in cell membranes do not redistribute. Considering the intensity never approached that of the insulin receptor, the mGrb10 SH2 domain (which is essentially identical to the hGrb-IR SH2 domain) appears to have a significantly lower affinity for the EGF receptor than the insulin receptor (7). hGrb-IR/Grb10 does not appear to bind IRS-1 (Fig. 6 and data not shown). Due to high backgrounds on the immunoblots, we have not been able to determine whether Grb-IR/Grb10 isoforms co-immunoprecipitate in 3T3-L1 adipocytes with activated insulin, PDGF, or EGF receptors. It has also been difficult to show co-immunoprecipitation of mGrb10 or Grb14 with activated PDGF and EGF receptors, even though these proteins are serine-phosphorylated in EGF- and PDGF-stimulated cells (9, 10). Grb10 does appear to bind the activated Ret cytoplasmic domain in vivo (12).

Intact hGrb-IR/Grb10 Binds More avidly Than Its Isolated SH2 Domain—To further differentiate potential mechanisms of interaction, comparisons were made using intact hGrb-IR/Grb10 and its isolated SH2 domain. Immobilized hGrb-IR/Grb10 precipitated insulin receptors from lysates of insulin-stimulated 3T3-L1 adipocytes, whereas receptors from unstimulated cells were not detected (Fig. 7A). In contrast, the immobilized hGrb-IR/Grb10 SH2 domain did not precipitate insulin receptors from these cells. Because many phosphoprotein-binding interactions are mediated by SH2 domains, and the mGrb10 SH2 domain (which is essentially identical to the hGrb-IR/Grb10 SH2 domain) binds insulin receptors from transfected cells (27), additional experiments were conducted with lysates from transfected HIRc fibroblasts. The full-length hGrb-IR/Grb10 protein was again found to precipitate activated insulin receptors (Fig. 7B). In addition, the immobilized...
SH2 domain precipitated significantly less but readily detectable amounts of insulin receptor. HIRc cells express considerably greater numbers of receptors per cell than differentiated 3T3-L1 adipocytes. Although higher receptor numbers may facilitate SH2 domain binding in the latter case, it certainly appears that in addition to its SH2 domain other regions of hGrb-IRβ/Grb10 participate in interactions with activated insulin receptors.

**Grb-IRβ/Grb10**

**Figure 6.** hGrb-IRβ/Grb10 binds insulin, EGF, and PDGF receptors. Differentiated 3T3-L1 adipocytes were stimulated or not (–) with insulin (I, 10⁻⁷ M for 5 min), EGF (E, 10⁻⁷ M for 5 min), or PDGF (P, 10 ng/ml for 10 min). Receptors were precipitated with immobilized GST or the hGrb-IRβ/Grb10 fusion protein and separated by SDS-PAGE. Alternatively, cell lysates (5% of the volume used in immunoprecipitations) were separated by SDS-PAGE. Proteins were identified by immunoblotting with anti-Tyr(P) antibodies.

**Figure 7.** hGrb-IRβ/Grb10 binds the insulin receptor more avidly than its isolated SH2 domain. After being stimulated with insulin (10⁻⁷ M for 5 min), 3T3-L1 adipocytes and HIRc fibroblasts were lysed. Proteins were precipitated with GST alone, GST-SH2 domain, or GST-hGrb-IRβ/Grb10, separated by SDS-PAGE, and identified by immunoblotting with anti-Tyr(P) antibodies.

**Figure 8.** Sites of hGrb-IRβ/Grb10 binding to the insulin receptor. Triton X-100-solubilized insulin receptors were activated with insulin and ATP. Wild-type (WT) receptors and three mutated forms were precipitated with GST-hGrb-IRβ/Grb10 (A) or GST-SH2 domain (B) and detected by anti-Tyr(P) immunoblotting. Receptor Y960F contains a single Tyr→Phe substitution within the juxtamembrane, PTB domain-binding site. ΔCT receptors lack 43 residues at the C terminus of the β subunits, including phosphorylation sites Tyr-1316 and Tyr-1322. All three activation loop tyrosines (1146, 1150, and 1151) are mutated to Phe in YF3 receptors.

**Additional Studies**

Additional studies tested the role of the hGrb-IRβ/Grb10 SH2 domain in mediating this interaction. The isolated SH2 domain precipitated equivalent amounts of activated, solubilized wild-type, Y960F, ΔCT, and YF3 receptors (Fig. 8B). These results support the conclusion that binding is not abolished by eliminating phosphorylation within any one of the three domains. Additional studies tested the role of the hGrb-IRβ/Grb10 SH2 domain in mediating this interaction. The isolated SH2 domain precipitated equivalent amounts of activated, solubilized wild-type, Y960F, ΔCT, and YF3 receptors (Fig. 8B). These results support the conclusion that binding is not abolished by eliminating phosphorylation within any one of the three insulin receptor domains and further demonstrate that the SH2 domain is involved in the interaction. These results suggest that hGrb-IRβ/Grb10 and its SH2 domain might bind the insulin receptor at more than one site, so elimination of any one site should not abolish binding. As an alternative explanation, hGrb-IRβ/Grb10 may bind the receptor at a previously unrecognized phosphorylation site (these exon 11 receptors should not be phosphorylated at sites proposed by Webster and colleagues (39)).

**Phosphopeptide Competition Studies**

To further investigate potential modes of interaction, synthetic phosphopeptides corresponding to each of the major insulin receptor phosphorylation domains were used to compete with interactions between wild-type insulin receptors and either intact hGrb-IRβ/Grb10 or its SH2 domain. The sequences include the monophosphoryl juxtamembrane site surrounding Tyr-960 (pY960), the bisphosphoryl C terminus encompassing Tyr-1316 and Tyr-1322 (pY2CT), and the triphosphoryl kinase activation loop (pY3Loop). These peptides were designed to bind SH2 domains and each extends at least 5 residues past the N- and C-terminal phosphotyrosine. Nevertheless, the peptides do not block inter-
actions between intact hGrb-IRβ/Grb10 and the insulin receptor, even at 1.0 mM concentrations (Fig. 9A). In contrast, SH2 domain binding was inhibited by two of the three peptides (Fig. 9B). Peptide pY3Loop had slightly higher inhibitory potency than pY960, whereas pY2CT had no effect. A previous study suggested that the mGrb10 SH2 domain binds the insulin receptor C terminus (27). Since the SH2 domain sequences of mGrb10 and hGrb-IRβ/Grb10 are essentially identical (Fig. 1A), results should be consistent. However, these investigators attempted to attach seven monophosphoryl peptides to Affi-Gel and use these reagents for pull-down studies. Due to large differences in chemical reactivity, levels of covalent peptide attachment typically vary many-fold. These levels were not determined. We conclude that hGrb-IRβ/Grb10 interacts with the insulin receptor via its SH2 domain and by an additional unidentified mechanism. The SH2 domain has potential for binding at least two sites, as has been shown previously for SH2 domain interactions with the EGF receptor (40). It appears to bind the phosphorylated insulin receptor kinase loop and juxtamembrane region with greatest avidity.

**SH3 Domain Binding to the 3BS Site of Grb-IRβ/Grb10**—hGrb-IRβ/Grb10, hGrb-IR, and mGrb10 have potential SH3 domain binding sites (Fig. 1A). To test whether interactions occur, immobilized SH3 domains from P1-3-kinase p85, Abl, and Fyn, and immobilized full-length Grb2 were used to precipitate proteins from Rat1 fibroblast lysates. Immunoblotting with anti-Grb10 antibodies revealed strong binding to the Abl SH3 domain but not the other proteins (Fig. 10). The negative results in these assays suggest that Grb-IR/Grb10 isotypes probably don’t bind P1-3-kinase p85, Fyn, or Grb2 via their SH3 domains in cells. While binding the Abl SH3 domain shows that Grb-IR/Grb10 isotypes can bind SH3 domain proteins, these results do not necessarily mean that this particular interaction has physiological relevance. The possibility that Grb-IR/Grb10 isotypes interact with c-Abl or the Abelson oncogene product is a subject for future investigation.

One potential SH3 domain binding site (3BS) is common to Grb7 and Grb14, in addition to the Grb-IR/Grb10 isoforms (Fig. 1A). A peptide corresponding to the 3BS site (SLPAIPNPFPEL) abolished binding to the Abl SH3 domain at a peptide concentration of 100 μM (Fig. 10). These findings strongly support the notion that SH3 domain proteins bind Grb-IR/Grb10 isoforms and that 3BS represents a site for binding.

**DISCUSSION**

We have identified a variant transcript of the human Grb-IR/Grb10 gene. The encoded protein has high sequence homology with hGrb-IR and mGrb10, although its domain architecture is more similar to that of Grb7 and Grb14. This is because hGrb-IR contains a 46-residue deletion within its PH domain and 58-residue extension at its N terminus, relative to our protein, Grb7 and Grb14. mGrb10 contains an 80-residue insertion, relative to the other proteins. The functions of these insertions, deletions, and extensions are unknown. We have analyzed functions of Grb-IR/Grb10 proteins in vitro and in cells. The protein is present in the cytosol of quiescent cells but translocates to the membrane upon insulin stimulation. This redistribution is likely through interactions with the insulin receptor. Receptor interactions with hGrb-IRβ/Grb10 are mediated in part through the SH2 domain, which binds to the phosphorylated kinase activation loop and the phosphorylated juxtamembrane region. This may explain why overexpression of Grb-IR inhibits insulin signaling (hGrb-IR and hGrb-IRβ/Grb10 SH2 domains are identical) (7). Binding at juxtamembrane Tyr-960 must block PTB domain interactions and prevent phosphorylation of substrates such as IRS-1 and Shc. Binding at the kinase loop would prevent substrate phosphorylation, as well.

It is also clear from our results that regions outside of the SH2 domain participate in receptor binding. The PH domain may have a role, perhaps analogous to the way tandem PH and PTB domains of IRS proteins participate in receptor recognition (5, 6, 29, 41, 42). However, it is difficult to predict the function of PH domains (26). This one is located within a longer (~320-residue) region of extended homology with the Grb7/Grb10/Grb14 family and the product of the Caenorhabditis elegans gene mig10 (9). This may suggest that the PH domain is embedded in a larger structure or flanked by one or two other functional domains. It has not been recognized previously that regions outside of the SH2 domains of Grb7, Grb10, Grb14, or Grb-IR participate in their interactions with phosphoproteins. Grb-IR lacks 40 residues of its apparent PH domain yet interacts avidly with the insulin receptor. This may imply that the PH is not involved. However, the N-terminal Grb-IR extension may compensate by completing the PH domain. Phospholipase C-β1 contains such a “split” PH domain with its entire SH2-SH2-SH3 domain region inserted between strands β3 and β6.
β of the PH domain β-sandwich. Strands β1–β3 are separated from the remainder of the domain by over 300 residues. Even though Grb-IR is a stable, folded protein, its PH domain appears to be missing strands β1–β3. Since one cannot generally remove large pieces of a stable protein fold without denaturing the protein and altering its physicochemical characteristics, something seems to have taken the place of strands β1–β3. The N-terminal extension would be one obvious candidate, although its sequence does not match the PH domain consensus.4

Our findings also show selective in vitro interactions between SH3 domains and Grb-IR/Grb10. We identified a high affinity site for c-Ab1 SH3 domain binding that is common to the known members of the Grb7/Grb10/Grb14/Grb-IR family. While further studies are needed to determine if these proteins bind c-Abl or the Abelson oncoprotein in cells, and which additional SH3 domain proteins bind Grb7/Grb10/Grb14/Grb-IR family members, it is tempting to speculate that these interactions might have potential roles in normal signaling and oncogenesis. Interestingly, the 3BS sequence (SLPAIPNPFPEL) does not conform to the known class I specificity of the Ab1 SH3 domain: (N)Px03XpXpXpC (θ and ψ represent residues with aromatic and hydrophobic side chains, respectively) (43, 44). If the orientation of 3BS is flipped, however, then it fits a class II aromatic and hydrophobic side chains, respectively) (43, 44). If that we report include the Northern analyses of hGrb7, the phospholipase C-α SH3 domains.

The interaction of Grb-IR/Grb10 with selected SH3 domains, with the insulin receptor at a site outside of its SH2 domain, though its sequencedoes not match the PH domain consensus.4 of the PH domain

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