Two Novel Proteins That Are Linked to Insulin-like Growth Factor (IGF-I) Receptors by the Grb10 Adapter and Modulate IGF-I Signaling*  

Barbara Giovannone†‡§, Eunhee Lee†§, Luigi Laviola, Francesco Giorgino, Kelly A. Cleveland‡, and Robert J. Smith†‡**

From the †Division of Endocrinology and the Hallett Center for Diabetes and Endocrinology, Rhode Island Hospital, Brown Medical School, Providence, Rhode Island 02903 and ‡Department of Emergency and Organ Transplantation, Internal Medicine, Endocrinology, and Metabolic Diseases, University of Bari, 70124 Bari, Italy

Grb10 is a protein that binds to the intracellular domains of activated tyrosine kinase receptors, including insulin-like growth factor (IGF-I) and insulin receptors. This occurs through the interaction of two C-terminal Grb10 motifs (BPS and Src homology domains) with receptor phosphotyrosine residues. Published data from transfection/overexpression studies support both positive and negative regulatory effects of Grb10, thus leaving its physiological role unclear. Because Grb10 has the structure of an adapter protein, the objective of this study was to determine whether Grb10 links other proteins to IGF-I receptors and thus modulates IGF-I signaling. Using yeast two-hybrid screening, the N terminus of Grb10 was shown to interact with two novel proteins, designated GIGYF1 (Grb10 interacting GYF protein 1) and GIGYF2. Mutation analysis indicates that a 17-amino acid sequence in GIGYF1 and GIGYF2, homologous to the GYF domain described previously, binds to tandem proline-rich regions in the N terminus of Grb10. In IGF-I receptor-expressing R+ fibroblasts, there is detectable binding of a Myc-tagged fragment of GIGYF1 to Grb10 in the basal state. Stimulation with IGF-I results in increased binding of GIGYF1 to Grb10 and transient binding of both Grb10 and GIGYF1 to IGF-I receptors, presumably via the adapter function of Grb10. At later time points, GIGYF1 dissociates, but Grb10 remains linked to IGF-I receptors. Overexpression of the Grb10 binding fragment of GIGYF1 in R+ cells results in a significant increase in IGF-I-stimulated receptor tyrosine phosphorylation. In conclusion, we have identified two members of a novel protein family, which become transiently linked to IGF-I receptors by the Grb10 adapter protein following IGF-I stimulation. Grb10 and GIGYFs may act cooperatively to regulate receptor signaling.

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The intracellular protein Grb10 is a member of a family of putative adapter proteins, which also includes Grb14 and Grb7. These proteins share common structural elements, including an N-terminal proline-rich region, a Ras-associating domain (1), a pleckstrin homology (PH) domain, a BPS (between PH and SH2) domain, and a C-terminal SH2 domain (2). Multiple isoforms derived from alternative splicing at the N terminus of Grb10 have been cloned from mouse, rat, and human cDNA libraries (3–10). Grb10 isoforms have been shown to bind to the intracellular domains of several activated tyrosine kinase receptors, including insulin, IGF-I (4–6, 11, 12), epidermal growth factor (5), and platelet-derived growth factor receptors (7), as well as the oncogenic tyrosine kinases BCR-Abl (14), Ret (15), and c-kit (16). Our previous work (6) has shown greater Grb10 binding to insulin versus IGF-I receptors, although significant interaction of Grb10 with both receptors can be demonstrated readily. This is consistent with data showing that the tandem BPS and SH2 domains are involved in the interaction with the insulin receptor, whereas only the BPS domain binds strongly to the IGF-I receptor (11, 12). The interaction of Grb10 with insulin and IGF-I receptors involves receptor phosphotyrosine residues and is dependent on receptor activation. Grb10 is not directly tyrosine-phosphorylated in response to hormone stimulation (17), but tyrosine phosphorylation of Grb10 has been reported as a result of its interaction with Tec (18) and Src (17). Grb10 proteins are serine-phosphorylated upon stimulation of cells with epidermal growth factor, platelet-derived growth factor, fibroblast growth factor, and insulin (3, 8), but the functional significance of this phosphorylation is still unknown.

Grb10 is believed to have a role in regulating cellular responses to insulin, IGF-I, and possibly other hormones. However, published studies on the effects of Grb10 overexpression in cultured cells have provided conflicting evidence for stimulatory versus inhibitory actions on mitogenesis and other hormone responses. For example, overexpression of human Grb10 in Chinese hamster ovary cells was shown to reduce both insulin-dependent phosphorylation of IRS-1 and activation of phosphatidylinositol 3-kinase (4). Data showing an inhibitory effect of Grb10α on IGF-I-stimulated mitogenesis, as well as a decrease in the catalytic activity of both insulin and IGF-I receptors (19), also have been reported (20). In contrast, other published work suggests positive effects of Grb10 on...
insulin and IGF-I action. Overexpression of a presumed dominant-negative fragment of Grb10, lacking the BPS-SH2 domain, partially inhibited cell growth stimulation by insulin and IGF-I in rat fibroblasts (5). In another study, a presumed dominant-negative SH2 domain fragment decreased cell proliferation, whereas overexpression of full-length Grb10 increased DNA synthesis upon growth factor treatment (21). The conflicting data do not appear to be attributable to the effects of different Grb10 isoforms or the properties of specific cell types and thus leave the physiological role of Grb10, as well its mechanism of action, unclear.

The SH2 domain of Grb10 has been reported to bind to some intracellular molecules such as mitogen-activated receptor 1, mitochondria-associated Raf (22, 23), and the ubiquitin protein ligase Nedd-4 (24), in addition to its interaction with receptor tyrosine kinases. However, there are no data on the functional significance of these interactions as they may relate to Grb10 effects on cell signaling. In a recent study, an interaction between Grb10 and Akt was demonstrated, suggesting a role for Grb10 in promoting Akt translocation to the plasma membrane in response to c-kit receptor tyrosine phosphorylation, thus augmenting Akt activity (16). The determinants of binding of Grb10 to Akt were not elucidated, although the PH and the SH2 domains were excluded.

Because Grb10 has the structure of an adapter protein, it has the potential to function as a bridge protein, with the C terminus bound to phosphorylated receptors and the N-terminal region bound to one or more other proteins. Using the yeast two-hybrid cloning system with the N-terminal portion of Grb10 as bait, we have identified two novel proteins that bind to Grb10, which we have designated GIGYF1 (Grb10 interacting Grb10–mammal interacting GYF protein 1) and GIGYF2. Studies on GIGYF1 demonstrate that it associates with Grb10 in cultured cells, becomes linked to activated IGF-I receptors, and modifies IGF-I signaling.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid Screening—The yeast strains L40 and AMR 70, the yeast expression vectors pVP16 and pBTM116, and 9.5- and 10.5-day mouse embryo cDNA libraries, plus a TA-1 fat cell library, in pVP16 were provided by Stanley Henggeln (Fred Hutchinson Cancer Research Center, Seattle, WA) (25, 26). The LexA fusion plasmid pBTM116 was constructed by Paul Bartel and Stanley Fields (27). To obtain a LexA-Grb10 hybrid construct, the mGrb10 full-length cDNA (6) was digested with EcoRI-SauI. The resulting 516-bp fragment (Grb10-NT), which includes the complete proline-rich region of Grb10, was subcloned into the yeast vector pBTM116.

The L40 yeast strain was transformed with pBTM116 containing the fused LexA-Grb10-NT coding sequence by a standard lithium acetate protocol (28, 29). After growth in Trp–medium, L40/pBTM116/Grb10-NT yeast cells were transformed with a pool of pVP16/9.5-day and pVP16/10.5-day mouse embryo cDNA libraries and, separately, with a TA-1 mouse fat cell cDNA library in aG1T11. Transformation with 500 μg of library plasmid DNA yielded ~12 × 10⁶ primary transformants, as confirmed by colony growth in Trp–Leu–medium. The yeast were subsequently grown in Trp–/Leu–/His–medium to 10 h to allow for the expression and formation of the transactivating LexA/VP16 complex, and a fraction of the yeast suspension (corresponding to ~4 × 10⁶ transformants) was plated on Trp–/Leu–/His–plates. Libraries clone showing activation of both reporter genes (HIS3 and LacZ) were tested in the two-hybrid system for specificity of association with the Grb10-NT using a LexA-lexA construct as a negative control. Plasmids from library clones were subsequently isolated from the yeast, transfected to Escherichia coli, sequenced, and used to search for similar sequences in GenBankTM.

Full-length GIGYF1 and GIGYF2 cDNA Cloning—Approximately 6 × 10⁵ plaques from a 15-day mouse embryo cDNA library in aG1T11 (Clontech, Palo Alto, CA) were screened with standard techniques using LexA as probes partial cDNAs of GIGYF1 and GIGYF2 obtained from the two-hybrid screening. Clones obtained by this method containing the largest inserts of GIGYF1 (lacking a portion of the 5′ coding region) and GIGYF2 (lacking both 5′ and 3′ coding sequences) were chosen for further study. The remaining 5′ coding sequence of GIGYF1 was obtained by reverse transcriptase PCR of mouse lung poly(A)+ RNA (Ambion, Austin, TX) using the Smart RACE cDNA amplification kit (Clontech, Palo Alto, CA), a forward adapter primer, and GIGYF1-specific reverse primer. The product was cloned into the pCR4-TOPO vector (Invitrogen) and sequenced in both directions. The missing 5′ end of GIGYF2 was amplified from mouse liver Marathon-Ready cDNA (Clontech) using the Smart RACE cDNA amplification kit as described for GIGYF1. The terminal 3′ coding sequence of GIGYF2 was amplified from mouse liver poly(A)+ RNA (Ambion) by Smart RACE, cloned into pCR4-TOPO, and sequenced in both directions.

Northern Blotting—GIGYF1 and GIGYF2 probes (571 and 388 bp, respectively) were generated by NorI excision of the GIGYF1 and GIGYF2 cDNAs from the pVP16 plasmids obtained from the two-hybrid screening and labeled with [32P] deoxy-CTP by random priming (multiple DNA labeling kit; Amersham Biosciences). A mouse multiple tissue poly(A)+ mRNA Northern blot (mouse MTN™ blot; Clontech, Palo Alto, CA) was hybridized overnight with radiolabeled probes at 65°C and washed twice with 2× SSC-0.5% SDS (30 min each) and once with 0.1× SSC-0.1% SDS, and specific mRNA bands were identified using a PhosphorImager (Amersham Biosciences).

Site-directed Mutagenesis—Deletion mutations of the proline-rich region of Grb10 and the GYF domain of GIGYF1 were generated using the ExSite PCR-based site-directed mutagenesis kit (Stratagene, La Jolla, CA) with 25–50-bp primers corresponding to the 5′ and 3′ ends of each proline-rich fragment or the GYF domain, respectively. The proline-rich box 1 deletion (aa 78–89), box 2 deletion (aa 94–102), and box 3 deletion (aa 106–113) were amplified using the appropriate site-specific primers with a fragment of Grb10 in pBluescript as a template. The double proline-rich box deletions 1.2 (aa 78–102), 1.3 (aa 78–89 and 106–113), and 2.3 (aa 94–113) were amplified using appropriate combinations of the same primers as the single box mutations and combinations of the same primers as the single box mutations and with single-deletion mutants as templates. The 17-aa GYF domain of GIGYF1 was amplified with specific primers using a fragment of GIGYF1 in pBluescript as a template. Point mutations of the 17-aa GYF region of GIGYF1 (W498A, G503A, Y504A, F560A) were generated with the QuikChange site-directed mutagenesis kit (Stratagene). The mutant plasmids were propagated using sequence-specific primers and the GIGYF1/pGilda construct as a template. All constructs were verified by automated sequencing.

Yeast Two-hybrid Interaction Analysis—Yeast two-hybrid assays were performed using the LexA-based Matchmaker kit (Clontech). GIGYF1 and GIGYF2 gene fragments were cloned into pGilda, and the Grb10 proline-rich domain (wild type) was cloned into pB42AD. The Grb10 and GIGYF1 mutants in pBluescript were subcloned into the pGilda yeast vector at EcoRIBamHI and, sequentially, into pB42AD at the EcoRIBamHI site. These constructs were transfected into the EGY48 yeast strain according to the manufacturer’s protocol. Interactions between the Grb10 proline-rich domain and GIGYF1 or GIGYF2 were determined using a selective medium agar plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal). A liquid β-galactosidase assay was performed on the co-transformed yeast cells, using o-nitrophenyl-β-D-galactopyranoside as substrate.

GIGYF1 Fragment Transfection—A fragment of GIGYF1 containing aa 410–598 with an N-terminal Myc tag was generated by PCR using a forward primer containing the 10-aa sequence EKQLSEEDL recognized by Myc monoclonal antibody (clone 9E10) (Roche Applied Science) and reverse primer containing specific GIGYF1 cDNA sequence with the largest of the initial GIGYF1 two-hybrid clones as template. The PCR product was cloned into the Xhol/XbaI sites of the pcDNA3 vector (from Dr. T. C. He, Howard Hughes Medical Institute, Baltimore, MD), subcloned into SmaI/EcoRI-cut and amplified from the liquid-nitrogen-preserved and then digested with EcoRI-XhoI receptor overexpressing fibroblasts (30) (provided by Dr. Renato Baserga, Jefferson Cancer Center, Thomas Jefferson University, Philadelphia, PA). Monolayers of R' cells (30–40% confluent) in 10-cm dishes were incubated for 6 h with 9 μg of purified pcDNA3/pcDNA3 and 30 μl of LipofectAMINE (Invitrogen) in 5.5 ml of OptiMem medium (Invitrogen) and then returned to standard growth medium consisting of Dulbecco’s modified Eagle’s medium with 4.5 g/liter glucose, 5% fetal bovine serum (Invitrogen), and 2.5 μg/ml hygromycin B (Sigma). The cells were studied 48 h post-transfection.

For stable transfection studies, a fragment of GIGYF1 containing an 410 to 598 was generated by PCR using a forward primer designed to amplify from a standard tumor cell line, U2-11, containing specific GIGYF1 cDNA sequence with the largest of the initial GIGYF1 two-hybrid clones as template. After cloning into the BamHI/HindIII sites of pcDNA 3.1 (Zeocin+) vector (Invitrogen) and sequence verification, the purified vector was transfected into R+ fibro-
Cloning of Two Grb10 Interacting Proteins

blasts using the LipofectAMINE protocol as described above. After growth for 30 days in the presence of Zeocin (100 μg/ml) (Invitrogen), clones were isolated by limiting dilution, tested for GIGYF1 expression by immunoblotting with Au1 monoclonal antibody (BabCO, Covance, Richmond, CA), and thereafter maintained in the presence of Zeocin (80 μg/ml).

Immunoprecipitation and Immunoblotting—Transfected R+ cells were incubated for 4 h in serum-free Dulbecco’s modified Eagle’s medium-H (4.5 g/liter glucose) containing 0.5% bovine serum albumin and 25 mM HEPES. IGF-I was added to the medium at a final concentration of 10−5 M, and the cells were incubated for 0, 2, or 10 min at 37 °C. The cells were washed with ice-cold buffer (137 mM NaCl, 1 mM MgCl2, 1 mM CaCl2, 0.1 mM Na3VO4, 20 mM Tris-HCl, pH 7.6) and lysed in the same buffer supplemented with 1% Nonidet P-40, 10% glycerol, 2 mM EDTA, 10 mM pyrophosphate, 10 mM NaF, 2 mM Na3VO4, 2 mM phenylmethylsulfonyl fluoride, and 8 μg/ml leupeptin. After clearing by centrifugation, protein concentration was determined by Bradford assay. To determine Grb10/GIGYF1 association, 0.5 μg of cell lysate was immunoprecipitated with anti-Grb10 antibody and blotted with anti-Myc antibody (Roche Applied Science). Phosphorylation of IGF-I receptors and IRS-1 was measured by direct blotting using anti-phosphotyrosine antibody (PY20) (Transduction Laboratories, San Jose, CA). Shc phosphorylation was examined by blotting with a polyclonal phosphospecific Shc antibody (UBI, Lake Placid, NY). Phosphorylation of ERK and Akt was measured using phosphospecific ERK antibody (from Dr. Laurie Goodyear, Joslin Diabetes Center, Boston, MA) and phosphospecific Akt antibody (New England Biolabs, Beverly, MA). Specific antibodies were used to measure the content of IGF-I receptors (31), IRS-1, and Shc (Transduction Laboratories, San Jose, CA) by direct immunoblotting. Specific protein bands were identified with 125I-protein A and quantified on a PhosphorImager or identified by ECL (PerkinElmer Life Sciences) and quantified on a densitometer.

Statistical Analysis—Data from densitometric analysis were adjusted to reflect percent stimulation of specific proteins in transfected cells at each time point with respect to empty vector transfected controls. All data are expressed as means ± S.E. and were subjected to a Kolmogorov-Smirnov test for normality, followed by paired t tests for comparisons between transfected cells and empty vector controls at each time point.

RESULTS

Two-hybrid Cloning of Proteins Binding the N-terminal Region of Grb10—A number of receptors and other tyrosine phosphoproteins have been identified that bind to the C-terminal region domains in Grb10. To search for proteins that bind to the N-terminal portion of Grb10 and thus may be linked to receptors by Grb10, yeast two-hybrid cDNA library screening was performed using as a bait an N-terminal fragment of mouse Grb10 that contains several proline-rich boxes but lacks the PH, BPS, and SH2 domains. The yeast vector pBTM116 expressing a fragment of mouse Grb10 (aa 1–172) as a fusion protein linked to the C terminus of the DNA binding domain of the transcription factor LexA was introduced into the yeast reporter strain L40. Yeast expressing the pLexA-Grb10 fragment fusion protein were then transformed with combined 9.5- and 10.5-day mouse embryo or differentiated TA-1 fat cell cDNA expression libraries in fusion with the VP16 transcriptional activation domain (25). With this system, positive clones are identified by detecting the activation of two reporter genes, HIS3 and LacZ, inserted into the L40 yeast genome with multiple LexA upstream activating sequences. A total of 64 and 78 yeast transformants were obtained from the embryo and fat cell libraries, respectively, representing 15 independent Grb10-interactive clones from the two libraries. Five of these clones consisted of partial sequences of two novel related proteins, which we have designated GIGYF1 and GIGYF2. GIGYF1 was encoded by three independent clones from the embryonic libraries and one clone from the fat cell library, whereas GIGYF2 was encoded by a single clone from the fat cell cDNA library. The GIGYF1 cDNA defined by the yeast two-hybrid clones represented a 571-bp ORF lacking a translation start site and stop codon. Similarly the GIGYF2 cDNA represented a 388-bp ORF lacking 5’ and 3’ terminal coding sequence. The remaining 10 clones were represented in single copy and were unrelated to GIGYF1 and GIGYF2.

Full-length Cloning and Structural Analysis of GIGYF1 and GIGYF2—An extended 2473-bp cDNA encoding an additional 594 bp of 5’ sequence and the complete 3’ sequence was obtained by screening a mouse 15-day embryo cDNA library using the largest GIGYF1 cDNA from the two-hybrid cloning as a probe. An overlapping clone containing the remaining 659 bp of 5’ coding sequence was generated from mouse lung poly(A)+ RNA by 5’-RACE PCR, and full-length GIGYF1 cDNA containing 9132 bp of coding sequence was assembled in pBluscript by restriction digestion-ligation of the two overlapping clones. The full-length GIGYF2 cDNA (3876 bp of coding sequence) was obtained in a similar manner. Screening of the mouse 15-day embryo cDNA library yielded a 2677-bp clone lacking both 5’ and 3’ terminal coding sequence. These segments were generated by 5’- and 3’-RACE PCR using mouse liver poly(A)+ RNA, and the fragments were assembled by restriction digestion-ligation of overlapping clones.

Analysis of nucleotide and amino acid sequences revealed that GIGYF1 and GIGYF2 are homologous proteins with common structural elements encoded by distinct genes (Fig. 1, A and B). Structural motifs present in both GIGYF1 and GIGYF2 include three potential tyrosine phosphorylation sites and candidate binding sites for protein phosphatase-1 (RVPF), 14—3—3 proteins (RSpSXP), and phospholipase Cγ (V(F)DDY). In addition, there is a proline-rich region in both proteins, a consensus bipartite nuclear localization motif, and a region of clathrin light chain homology (Fig. 1B). A striking feature is multiple stretches of Gln and Glu residues in the C-terminal half of both proteins (Fig. 1A).

Using the cloned cDNAs, mouse genomic sequences corresponding to both GIGYF1 and GIGYF2 were identified in GenBank™. The GIGYF1 gene mapped to mouse chromosome 5 as a relatively small gene (~7000 bp) with a coding sequence of 3 kb composed of 24 exons and very short introns. Our cloned GIGYF1cDNA corresponds to an ORF that has been designated Perq1 (XM_124667) based on its high abundance of Pro, Glu, Arg, and Gln (32). There are several single nucleotide differences in the cloned GIGYF1 in comparison with the predicted ORF, three of which would result in amino acid sequence substitutions. The GIGYF1 cDNA that we have cloned includes an additional 30 aa extending N-terminally from the putative start site of the Perq1 protein proposed on the basis of genomic sequence. This region is encoded by an ORF that includes an addition 5’ exon and starts with a strong Kozak consensus ATG (ANATGC) (33). The cloned cDNA also includes a run of 12 CAG triplets in the C-terminal region, whereas only nine CAG repeats are noted in the comparable region of genomic sequence. Although a homologous Perq1 gene product (NM_022574) has been predicted by the analysis of human chromosome 7q22 (34) and both human (AK001739 and AB014542) and chicken (U90567) EST sequences, there are no published data on the expression or functional properties of Perq1.

The GIGYF2 gene is located on mouse chromosome 1 (contig NW_000169), although the exact chromosomal location is still ambiguous. GIGYF2 is encoded by 27 exons and 26 large introns. Despite a high degree of homology in sequence and exon structure in GIGYF1 and GIGYF2, the GIGYF2 gene spans a much larger region of gene sequence (~90,000 versus ~7000 bp of GIGYF1). The GIGYF2 cDNA that we have cloned encodes a protein identical to a predicted gene product described as similar to TNRC15, which has been identified in mouse genomic.
**FIG. 1.** Sequence and structural motifs of mGIGYF1 and GIGYF2. A, alignment of mGIGYF1 and mGIGYF2 aa sequences. The underlined sequences represent the original two-hybrid clones. The Grb10 binding site (GYF domain) is outlined by the shaded box, and stretches of Gln and Glu residues are shown in bold. B, diagram indicating consensus motifs present at comparable sites in GIGYF1 and GIGYF2.
contig NW_000169. Partial cDNAs encoding this sequence have been identified from at least two different mRNA sources (BC027137 and BC030845). The homologous human GIGYF2 gene is located on chromosome 2q36.1 and was designated TNRC15 (trinucleotide repeat containing 15) (locus identification 26058). Evidence for mRNA expression has been obtained through a study screening for large proteins in human brain (35).

**Tissue Distribution of Mouse GIGYF1 and GIGYF2**—Northern blotting experiments on multiple mouse tissues were performed to examine the distribution of GIGYF1 and GIGYF2 mRNA. As shown in Fig. 2A, a single band of ~6 kb corresponding to the GIGYF1 message was detectable in all of the tissues tested. The highest levels of mRNA were seen in brain and lung, with heart, spleen, and kidney also exhibiting comparatively high levels. GIGYF1 mRNA is poorly expressed in liver and testis and very low in skeletal muscle, where only a faint band was detected. When the same tissues were probed for GIGYF2, a 7-kb transcript was detected. Expression levels are highest in heart and liver, somewhat lower in kidney and testis and very low in skeletal muscle, where only a faint band was detected. When the same tissues were probed for GIGYF1, a ~1.5-kb band in these same tissues resulted in a total loss of binding to the proline-rich region of Grb10, as determined by two-hybrid plaque assays (not shown) and liquid (spectrophotometric) assays. The sequence of the 17-aa motif in T-lymphocyte CD2-binding protein 2 (CD2BP2) was shown to mediate the association of two proline-rich boxes in CD2 with CD2BP2 (37).

**Analysis of Structural Requirements for Grb10 Binding**—The only candidate protein-protein interaction motif identified in the fragment of Grb10 used in the two-hybrid cloning of the GIGYF proteins consisted of three blocks of proline-rich sequence. Analysis of the GIGYF1 and GIGYF2 sequences revealed a region with high homology to the GYF domain described recently (Fig. 3A) (36). This 17-aa motif in T-lymphocyte CD2-binding protein 2 (CD2BP2) was shown to mediate association of two proline-rich boxes in CD2 with CD2BP2 (37). To determine its role in GIGYF binding to Grb10, a pGilda yeast two-hybrid vector was prepared containing sequence equivalent to the initial GIGYF two-hybrid clone from which the entire 17-aa GYF was deleted by excision mutagenesis. This resulted in a total loss of binding to the proline-rich region of Grb10, as determined by two-hybrid plaque assays (not shown) and liquid-phase assays (Fig. 3B). To further investigate the association of the GYF domain of GIGYF1 with Grb10, four mutant constructs were generated with Ala substituted for a single aa shown previously (37) to be critical for the interaction of CD2BP2 with CD2. As shown in Fig. 3B, the W489A, G503A, and Y504A substitutions within the GYF domain resulted in a marked decrease in binding of Grb10 to GIGYF1 and a significant decrease in GIGYF2 binding to Grb10 but no change in GIGYF2 binding to Grb10 but no change in GIGYF1 binding. Deletion of box 1 (SLPAIPNPFPPEL) resulted in a modest but significant decrease in GIGYF2 binding to Grb10 but no change in GIGYF1 binding. Deletion of box 3 (PPPPSQPPP) resulted in a significant decrease in binding of Grb10 to GIGYF1 and a suggestive decrease in GIGYF2 binding, which did not reach statistical significance. Excision of any two proline-rich boxes led to an essentially complete loss of Grb10 binding to GIGYF1 and GIGYF2. The data indicate distinct influences of individual proline-rich domains on Grb10-GIGYF binding and a critical requirement for the presence of at least two of the three proline-rich sequences.

**Grb10-GIGYF1 Interaction in Intact Cells**—To assess the interaction of GIGYF1 with Grb10 in intact cells, a Myc-tagged fragment of GIGYF1 corresponding to the sequence originally cloned by two-hybrid screening was generated in the pAdTrack vector and transiently transfected into R+ fibroblasts, a cell line expressing a high number of IGFI receptors (approximately ~1 x 10^6/cell). Grb10 association with GIGYF1 was assessed by immunoprecipitation with Grb10 antibody and immunoblotting with Myc antibody. Cells transfected with empty vector were used as control. Binding of the transfected Myc-GIGYF1 fragment to Grb10 was evident in the basal state, increased markedly 2 min after IGFI stimulation, and then became essentially undetectable 10 min after IGFI (Fig. 5A). Quantitative analysis of three independent experiments indicated that the fraction of total cellular GIGYF1 fragment associated with Grb10 increased ~3.5-fold after IGFI-I treatment from 0.9% of the
total GIGYF1 fragment in the basal state to 3.4% at 2 min after IGF-I. Because partial dissociation of GIGYF1 from Grb10 likely occurred during the immunoprecipitation procedure, this represents a minimum estimate of the fraction of GIGYF1 associated with Grb10 as a consequence of IGF-I stimulation. Co-precipitation of GIGYF1 with IGF-I receptors was demonstrated 2 min after IGF-I stimulation but undetectable in the basal state and 10 min after IGF-I (Fig. 5B).

As described previously in our laboratory (6), the co-precipitation of Grb10 with IGF-I receptors was absent in the basal state, rapidly stimulated by IGF-I, and persisted for >20 min (data not shown). These data are consistent with the following: 1) GIGYF1 binding to Grb10 in the basal state, 2) increased GIGYF1-Grb10 binding and recruitment to the GIGYF1-Grb10 complex to the activated IGF-I receptor, and 3) dissociation of GIGYF1 from a persistent Grb10-IGF-I receptor complex after several minutes.

**GIGYF1 Effects on IGF-I Signaling**—To evaluate the effects of GIGYF1 on IGF-I signaling, the same GIGYF1 fragment used in the co-precipitation studies described above was AU1-tagged and cloned into the pcDNA 3.1 vector. After transfection into R+ fibroblasts, multiple cell clones stably overexpressing similar levels of GIGYF1 were obtained (Fig. 6A). Signaling responses to IGF-I were compared in these cells and R+ fibroblasts transfected with empty pcDNA 3.1 vector. Overexpression of the GIGYF1 fragment had no effect on basal IGF-I receptor tyrosine phosphorylation but resulted in an ~60% increase in receptor phosphorylation 2 min after IGF-I stimulation and a less but still significant (41%) increase 10 min after IGF-I (Fig. 6, B and C). A similar increase in tyrosine phosphorylation of two cellular signaling proteins immediately downstream from the IGF-I receptor also was observed with GIGYF1 fragment overexpression. IRS-1 tyrosine phosphorylation was unaffected in the basal state and increased 50% at 2 min and 40% at 10 min after IGF-I in GIGYF1 overexpressing cells (Fig. 7, A and B). Tyrosine phosphorylation of the p52 Shc isoform was unaltered under basal conditions and increased by

![Fig. 4. Analysis of interaction between the mutated proline-rich domain of Grb10 and intact GYF of GIGYF1. Wild-type (WT) GIGYF1 GYF-like domain was cloned into the pGilda vector, and Grb10 proline-rich domain wild-type and deletion mutants were cloned into the pB42AD vector. These constructs were transformed into EGY48 yeast, which were pre-transformed with a LacZ encoding vector (p8op-LacZ). Interactions were measured by LacZ screening in plaque assays (blue-white screening; data not shown) and liquid (spectrophotometric) assays. The sequence of the Grb10 proline-rich domain with the deleted proline-rich boxes highlighted in bold print is depicted in panel A. The results of the liquid spectrophotometric assay of Grb10 proline-rich box interactions with GIGYF1 and GIGYF2 are listed in graphical form in panels B and C, respectively. Data are means ± S.E. obtained from four independent clones analyzed in duplicate. **, p < 0.001; *, p < 0.05.**

![Fig. 5. Co-precipitation of Grb10, GIGYF1, and IGF-I receptors in R+ fibroblasts. R+ fibroblasts were transiently transfected with a Myc-GIGYF1 fragment containing the Grb10 binding region. Empty vector (pAdTrack) was also transfected as a control. After 48 h, the cells were serum-deprived and stimulated with IGF-I (10⁻⁷ M) for 0, 2, and 10 min. Cell lysates (2 mg) were immunoprecipitated with Grb10 antibody (A) or IGF-I receptor antibody (B) and blotted with anti-Myc antibody. The level of Myc-GIGYF1 in transfected cells is indicated in the first lane in panel A, which represents an immunoblot of 200 μg of total cell lysate protein (not subjected to immunoprecipitation). The immunoblots are representative of three independent experiments.

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![Fig. 6. Cloning of Two Grb10 Interacting Proteins](http://www.jbc.org/Downloaded from)
Cloning of Two Grb10 Interacting Proteins

**DISCUSSION**

Although the function of Grb10 has not been established clearly, evidence from multiple studies indicates that Grb10, as well as other members of this putative adapter protein family (Grb7 and Grb14), may have important roles in modulating cellular signaling responses to receptor tyrosine kinases (3-7, 11–16). The use of overexpression studies to elucidate the physiological actions of adapter proteins, which serve to recruit other proteins into complexes, may be especially difficult, and this may explain conflicting published data on Grb10 (4, 5, 19–21). Depending on the levels of adapter protein expression and the extent of recruitment of effector proteins to receptors in untransfected cells, adapter protein overexpression could result in either increased formation or competitive dissociation of effector-adapter-receptor complexes. As an alternative approach to investigating Grb10 function, we used yeast two-hybrid cloning methods to search for proteins that bind to N-terminal domains in Grb10 and, thus, may be linked to receptor tyrosine kinases through their established interactions with C-terminal domains in Grb10. With a bait construct corresponding to the region of mGrb10 extending from the N terminus to just upstream from the PH domain, thus excluding the receptor-binding BPS and SH2 domains, we screened mouse embryo and fat cell expression libraries. This resulted in the identification of a novel family of two proteins, GIGYF1 and GIGYF2, which are encoded by distinct genes but share a 17-aa GYF domain was described initially as a proline-rich sequence homology and an overall aa homology of ~40%. The 17-aa GYF domain was described initially as a proline-rich interactive motif in CD2BP2 (37), and, based on crystal structure data, it has been proposed that a bulge-helix-bulge conformation of the GYF domain interacts with paired proline-rich sequences (36). Using mutant constructs of GIGYF1, we have shown that its interaction with Grb10 in two-hybrid assays exhibits sequence requirements similar to those essential for the binding of the CD2BP2 GYF domain to CD2. Although the specific Grb10 proline-rich sequences are distinct from the GYF-interactive sequences in CD2, our two-hybrid analyses of Grb10 deletion mutants have shown a similar requirement for two proline-rich motifs separated by a six- to seven-aa segment.

Thus, our data support the general interaction model proposed

![Figure 6: Effects of overexpression of GIGYF1 fragment on IGF-I phosphorylation](image)

**Fig. 6. Effects of overexpression of GIGYF1 fragment on IGF-I phosphorylation.** R+ fibroblasts were transfected with an AUI-GIGYF1 fragment containing the Grb10 binding region or with pcDNA3 empty vector and selected with Zeocin antibiotic (100 μg/ml). GIGYF1 fragment expression level was measured by direct immunoblotting with anti-AU1 antibody, and three clones expressing similar levels of the transfected protein were chosen (A). These cells, plus control cells transfected with the empty vector, were stimulated with IGF-I (10-7 M) for 0, 2, or 10 min after overnight serum deprivation. Proteins (60 μg) derived from cell lysates were resolved by SDS-PAGE (8.5%) and immunoblotted with anti-phosphotyrosine antibody (PY20) or anti-IGF-I receptor antibody (B, upper panel). IGF-I receptor phosphorylation from the three cell clones in four independent experiments normalized as percent empty vector control was quantified by densitometry (C). Data are means ± S.E., n = 12. **p < 0.001; *, p < 0.05.

![Figure 7: Effects of overexpression of GIGYF1 fragment on IRS-1 phosphorylation](image)

**Fig. 7. Effects of overexpression of GIGYF1 fragment on IRS-1 phosphorylation.** Stably transfected R+ fibroblasts expressing the AUI-GIGYF1 fragment containing the Grb10 binding region were stimulated with IGF-I (10-7 M) for 0, 2, or 10 min after overnight serum deprivation. Cells transfected with pcDNA3 empty vector were used as control. Proteins (80 μg) derived from cell lysates were resolved by SDS-PAGE (8.5%) and immunoblotted with anti-phosphotyrosine antibody (PY20) or anti-IRS-1 (A, upper panel) or anti-IRS-1 (A, lower panel). IRS-1 phosphorylation from three cell clones in four independent experiments normalized as percent empty vector control was quantified by densitometry (B). Data are means ± S.E., n = 12. **p < 0.001; *, p < 0.05.
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by Reinherz and co-workers (36) but define additional proline-rich sequences capable of GYF domain binding.

In an earlier report on the CD2BP2 protein, partial human sequences corresponding to GIGYF1 and GIGYF2 were recognized in GenBank™ (36). Using the complete mouse cDNAs that we have cloned, analysis of the human genome sequence and available DNA sequences from multiple other species indicate that GIGYF1 and GIGYF2 are the only two members of this protein family. Based on multi-tissue poly(A)/H11001 Northern blots, GIGYF1 and GIGYF2 mRNAs have distinct patterns of expression in specific mouse tissues. For example, GIGYF1 mRNA is most abundant in brain, spleen, lung, and kidney, whereas GIGYF2 has highest abundance in heart and liver. Grb10 has been shown previously (3) to have broad tissue expression in the mouse, and, in general, mRNAs for either GIGYF1, GIGYF2, or both of these proteins are present in all tissues expressing Grb10. One exception appears to be skeletal muscle, which has abundant Grb10 mRNA but very low levels of GIGYF1 and GIGYF2 mRNA.

Interactions of a transfected Myc-tagged GIGYF1 fragment containing the Grb10 binding region with Grb10 and IGF-I receptors were investigated in the R+ fibroblast cell line. In cells deprived of serum overnight and incubated in the absence of IGF-I a modest level of Grb10 co-precipitation with GIGYF1 was evident, but there was no detectable co-precipitation of either Grb10 or GIGYF1 with the IGF-I receptor. Within 2 min of IGF-I stimulation, there was rapid recruitment of Grb10 to the IGF-I receptor, a marked increase of the amount of GIGYF1 associated with Grb10, and a coordinate co-precipitation of GIGYF1 with the receptor. These data are consistent with the previously described recruitment of Grb10 to activated (tyrosine-phosphorylated) IGF-I receptors through receptor interactions with the Grb10 BPS domain (11). The co-precipitation of GIGYF1 with activated IGF-I receptors most probably reflects the formation of a trimeric complex, with the C-terminal region of Grb10 linked to the receptor and the N-terminal region bound to GIGYF1, because the Grb10 domain is the only apparent protein interaction motif in the transfected fragment of GIGYF1, and the IGF-I receptor lacks paired proline-rich motifs, which might function as direct GIGYF1 binding sites. We speculate that increased binding of GIGYF1 to Grb10 occurs as a consequence of Grb10 recruitment to the activated receptor, because effects of IGF-I on Grb10 must be receptor-mediated. It is not yet clear whether the observed level of Grb10 asso-

![Fig. 8. Effects of overexpression of GIGYF1 fragment on Shc phosphorylation.](#)

Stably transfected R+ fibroblasts expressing the AU1-GIGYF1 fragment containing the Grb10 binding region were stimulated with IGF-I (10^{-7} M) for 0, 2, or 10 min after overnight serum deprivation. Cells transfected with pcDNA3.1 empty vector were used as control. Proteins (60 µg) derived from cell lysates were resolved by SDS-PAGE (10%) and immunoblotted with anti-phospho-Shc (A, upper panel) or anti-Shc (A, lower panel). Shc phosphorylation from three cell clones in four independent experiments normalized as percent empty vector control was quantified by densitometry (B). Data are means ± S.E., *p < 0.05, **p < 0.001.

![Fig. 9. Effects of overexpression of GIGYF1 fragment on Akt and ERK phosphorylation.](#)

Stably transfected R+ fibroblasts expressing the AU1-GIGYF1 fragment containing the Grb10 binding region were stimulated with IGF-I (10^{-7} M) for 0, 2, or 10 min after overnight serum deprivation. Cells transfected with pcDNA3.1 empty vector were used as control. Proteins (60 µg) derived from cell lysates were resolved by SDS-PAGE (10%) and immunoblotted with anti-phospho-Akt (A, upper panel) or anti-phospho-ERK (B, upper panel). Akt phosphorylation (A, lower panel) and ERK phosphorylation (B, lower panel) from three cell clones in four independent experiments normalized as percent empty vector control was quantified by densitometry. Data are means ± S.E., *p < 0.05, **p < 0.001.
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Further studies will be required to determine the mechanism of GIGYF1 effects on IGF-I receptors. In initial studies with NIH-3T3 fibroblasts expressing lower levels of GIGYF1 and IGF-I receptors, augmented IGF-I signaling was not evident (data not shown). It therefore will be important to examine the roles of receptor, Grb10, and GIGYF1 abundance in the regulatory effects of GIGYF1 on IGF-I receptor signaling. However, the observed augmentation of IGF-I signaling by the GIGYF1 fragment in R+ fibroblasts indicates a potentially important role for the GIGYF proteins in the transmission of intracellular signals from IGF-I receptors and possibly from other receptor tyrosine kinases that bind Grb10.

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Two Novel Proteins That Are Linked to Insulin-like Growth Factor (IGF-I) Receptors by the Grb10 Adapter and Modulate IGF-I Signaling
Barbara Giovannone, Eunhee Lee, Luigi Laviola, Francesco Giorgino, Kelly A. Cleveland and Robert J. Smith

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