Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 10287–10291, October 1995
Biochemistry

Grb-IR: A SH2-domain-containing protein that binds to the insulin receptor and inhibits its function

(phosphatidylinositol 3-kinase/yeast two-hybrid system/insulin-receptor substrate 1)

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Communicated by Ellis Engelesberg, University of California, Santa Barbara, CA, July 25, 1995 (received for review June 19, 1995)

ABSTRACT **To identify potential signaling molecules involved in mediating insulin-induced biological responses, a yeast two-hybrid screen was performed with the cytoplasmic domain of the human insulin receptor (IR) as bait to trap high-affinity interacting proteins encoded by human liver or HeLa cDNA libraries. A SH2-domain-containing protein was identified that binds with high affinity *in vitro* to the autophosphorylated IR. The mRNA for this protein was found by Northern blot analyses to be highest in skeletal muscle and was also detected in fat by PCR. To study the role of this protein in insulin signaling, a full-length cDNA encoding this protein (called Grb-IR) was isolated and stably expressed in Chinese hamster ovary cells overexpressing the human IR. Insulin treatment of these cells resulted in the *in situ* formation of a complex of the IR and the 60-kDa Grb-IR. Although almost 75% of the Grb-IR protein was bound to the IR, it was only weakly tyrosine-phosphorylated. The formation of this complex appeared to inhibit the insulin-induced increase in tyrosine phosphorylation of two endogenous substrates, a 60-kDa GTPase-activating-protein-associated protein and, to a lesser extent, IR substrate 1. The subsequent association of this latter protein with phosphatidylinositol 3-kinase also appeared to be inhibited. These findings raise the possibility that Grb-IR is a SH2-domain-containing protein that directly complexes with the IR and serves to inhibit signaling or redirect the IR signaling pathway.**

In recent years extensive progress has been made in our understanding of how the insulin receptor (IR) and other receptor tyrosine kinases signal subsequent cellular biological responses. A major advance has been the identification of a particular amino acid sequence motif (called the SH2 domain) that binds to tyrosine-phosphorylated proteins with relatively high affinity (1). The specificity of these SH2 domains varies depending upon the amino acid residues immediately surrounding the phosphotyrosine [Tyr(*P*)]. Some of these SH2-domain-containing proteins [such as the SH2-domain-containing phosphatidylinositol (PI) 3-kinase and the tyrosine phosphatase SH-PTP2] have an intrinsic enzymatic activity that is activated upon binding to a tyrosine-phosphorylated protein. Others serve to link tyrosine-phosphorylated proteins to another protein with enzymatic activity (for example, Grb-2 links tyrosine-phosphorylated proteins to a guanine-nucleotide-release protein for Ras called SOS) (1). In the case of most receptors with tyrosine kinase activity, multiple SH2-domain-containing proteins have been identified that bind to these autophosphorylated receptors with high affinity (2). In contrast, for insulin and insulin-like growth factor I receptors, these same SH2-domain-containing proteins only weakly bind to these receptors. However, a cytoplasmic substrate of the IR tyrosine kinase, called IR substrate 1 (IRS-1), has been identified that binds to these SH2-domain-containing proteins with high affinity (3). Whether the binding of a SH2-domain-containing protein whose function is mediated at the plasma membrane (for example, the PI 3-kinase or the ras exchange protein) to a tyrosine-phosphorylated cytosolic protein is sufficient to trigger a biological response is still unclear, although several types of experimental approaches have indicated a role for IRS-1 in mediating various subsequent biological responses (4, 5).

To further identify signaling molecules involved in insulin action, we have utilized the yeast two-hybrid system (6) to find proteins that interact with the cytoplasmic domain of the IR. In the present report, we describe one such protein, called Grb-IR,[†] identified in this screen. Stable cell lines overexpressing this protein have been used to show that this protein, to our knowledge unlike any other previously described SH2-domain-containing protein, binds with high stoichiometry *in situ* to the phosphorylated IR. Moreover, overexpression of this protein was found to inhibit the tyrosine phosphorylation of two endogenous substrates of the IR kinase. These results suggest that this protein may either inhibit signaling by the IR or redirect the signaling to another pathway.

MATERIALS AND METHODS

Yeast Two-Hybrid Screen. A 1.2-kb *Eco*RI–*Xba* I cDNA fragment encoding the cytoplasmic domain of human IR was generated by PCR and fused to the sequence encoding the Gal4 DNA binding domain in plasmid pGBT9 (Clontech). The recombinant plasmid pGBT9-IRc was cotransformed into yeast host cell HF7C with two-hybrid libraries derived from either liver or HeLa cell cDNA (Clontech). Plasmids isolated from His- and β-galactosidase-positive clones were used to transform *Escherichia coli* HB101. To eliminate false positives, the plasmids isolated from bacteria were used to cotransform yeast strain SFY526 with plasmid pGBT9, pLAM5, an unrelated recombinant plasmid, or pGBT9-IRc, the plasmid encoding the cytoplasmic domain of human IR, respectively. Transformants were grown in synthetic medium containing 2% (wt/vol) glucose without Leu or His or both. Positive clones were identified by β-galactosidase activity filter assay.

Nucleotide Sequencing, RNA Analysis, and cDNA Cloning. The nucleotide sequences were determined from both directions by the dideoxynucleotide chain-termination method (7). Homology searches, sequence comparisons, and alignments were done with IG programs (IntelliGenetics). A human-tissue Northern blot (Clontech) was hybridized under stringent conditions with the random hexamer radiolabeled 0.9-kb DNA fragment isolated from the two-hybrid screen. The full-length

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Abbreviations: IR, insulin receptor; GAP, GTPase-activating protein; Tyr(*P*), phosphotyrosine; PI, phosphatidylinositol; IRS-1, insulin-receptor substrate 1; GST, glutathione *S*-transferase; HA, hemagglutinin; EGF, epidermal growth factor; PH, pleckstrin homology domain; WGA, wheat germ agglutinin.

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[†]The sequence reported in this paper has been deposited in the GenBank data base (accession no. U34355).

cDNA encoding Grb-IR was obtained by screening an oligo(dT)-primed human muscle cDNA library (Stratagene) with the same probe used for Northern blot analysis. Total RNA from HeLa cells was isolated by using the RNA STAT-60 kit (TEL-TEST "B;" Tel-Test, Friendswood, TX) and transcribed into cDNA by using the reverse transcription-coupled PCR kit (Stratagene). PCR-ready human fat cDNA was from Clontech. The primers used for PCR studies were as follows: P1, 5′-TTGAAGAAGGCAGAAGGAACCC-3′ (nt 210–230 in Grb-IR); P2, 5′-CATTTGACTGCTGGCACC-3′ (nt 1054–1033 in Grb-IR); P3, 5′-CAACGATATTAACTCGTCCGTG-3′ (nt 416–438 in Grb10); P4, 5′-CAAAAGTCACTGTGTGGA-3′ (nt 828–846 in Grb-IR); P5, 5′-CTCCTTTGTTCAGCTGGT-3′ (nt 1831–1814 in Grb-IR). PCR was carried out with *Taq* DNA polymerase (BRL).

Immunoprecipitations and Western Blot Analyses. Cells were lysed in lysis buffer containing 50 mM Hepes (pH 7.6), 1 mM EDTA, 1 mM EGTA, 150 mM NaCl, 1% Triton X-100, 10 mM NaF, aprotinin (10 μg/ml), leupeptin (10 μg/ml), 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, and 20 mM sodium pyrophosphate. Cell lysates or immunoprecipitates were separated by SDS/PAGE in 10% polyacrylamide gels and then examined by immunoblot analysis as described (8). The polyclonal antibody to Grb-IR was generated against the purified SH2 domain containing glutathione *S*-transferase (GST) fusion protein (GST–Grb-IRc) described below. Before use, the antibodies were affinity-purified on a column containing immobilized purified fusion protein and depleted of antibodies to the GST protein.

***In Vitro* Binding of IR with GST–Grb-IR Fusion Protein.** The *Eco*RI fragment encoding the C terminus of Grb-IR (residues 369–548) was excised from the two-hybrid-positive clone and inserted into a bacterial expression vector, pGEX-4T-3 (Pharmacia). Expression and purification of the GST fusion protein GST–Grb-IRc was carried out as described by the manual from Pharmacia. Cell lysates from CHO-IR and A431 cells were incubated with GST–Grb-IRc fusion protein (≈1 μg) bound to glutathione-agarose beads (Sigma) for 4 h at 4°C, washed three times in WG buffer (50 mM Hepes, pH 7.6/0.15 M NaCl/0.1% Triton X-100), and boiled for 5 min in SDS sample buffer, and the eluted proteins were examined by Western blot analysis.

Overexpression of Grb-IR in CHO-IR Cells and *in Situ* Association of Grb-IR with IR. The entire coding region of Grb-IR except the last two amino acids was amplified by PCR and fused in-frame at its 3′ end with a sequence encoding the 9-amino acid epitope hemaglutinin (HA) tag (YPYDVPDYA) by insertion into the expression vector pBEX-1 (9). CHO-IR cells were transfected with the recombinant plasmid (20 μg) and the puromycin-resistance vector PBSpacDp (1 μg) (10) by the calcium phosphate method (8). Transfectants were selected with puromycin (8 μg/ml) and colonies were screened by immunoblot analysis with anti-HA antibodies (Berkeley Antibody, Richmond, CA).

PI 3-Kinase Assay. Cell lysates were immunoprecipitated with anti-Tyr(*P*) antibody (py20) bound to protein G-Sepharose beads (Pharmacia) for 4 h at 4°C. The precipitates were assayed for PI 3-kinase activity as described (8).

RESULTS AND DISCUSSION

Identification of the Grb-IR cDNA. To find proteins that interact with the cytoplasmic domain of the IR, we constructed a plasmid in which the gene encoding the IR cytoplasmic domain (aa 958–1355) was fused to the sequence encoding the Gal4 DNA binding domain. In agreement with the studies of O'Neill *et al.* (11), this construct resulted in the production of a tyrosine-phosphorylated IR β subunit in the yeast (data not shown). By using the IR cytoplasmic domain as a bait, we screened yeast two-hybrid libraries derived from liver and HeLa cell cDNA. From 6 million colonies screened, we obtained 21 positive colonies that grew on minimum medium lacking Leu, Trp, and His and were positive for β-galactosidase activity by colony filter assays.

A

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        10         20         30         40         50         60         70
MALAGCPDSF LHHPYYQDKV EQTPRSQQDP AGPGLPAQSD RLANHQEDDV DLEALVNDMN ASLESLYSAC

        80         90        100        110        120        130        140
SMQSDTVPLL QNGQHARSQP RASGPPRSIQ PQVSPRQRVQ RSQPVHILAV RRLQEEDQQF RTSSLPAIPN

       150        160        170        180        190        200        210
PFPELCGPGS PPVLTPGSLP PSQAAAKQDV KVFSEDGTSK VVEILADMTA RDLCQLLVYK SHCVDDNSWT

       220        230        240        250        260        270        280
LVEHHPHLGL ERCLEDHELV VQVESTMASE SKFLFRKNYA KYEFFKNPMN FFPEQMVTWC QQSNGSQTQL

       290        300        310        320        330        340        350
LQEPRHLQLL ADLEDSNIFS LIAGRKQYNA PTDHGLCIKP NKVRNETKEL RLLCAEDEQT RTCWMTAFRL

       360        370        380        390        400        410        420
LKYGMLLYQN YRIPQQRKAL LSPFSTPVRS VSENSLVAMD FSGQTGRVIE NPAEAQSAAL EEGHAWRKRS

       430        440        450        460        470        480        490
TRMNILGSQS PLHPSTLSTV IHRTQHWFHG RISREESHRI IKQQGLVDGL FLLRDSQSNP KAFVLTLCHH

       500        510        520        530        540
QKIKNFQILP CEDDGQTFFS LDDGNTKFSD LIQLVDFYQL NKGVLPCKLK HHCIRVAL.
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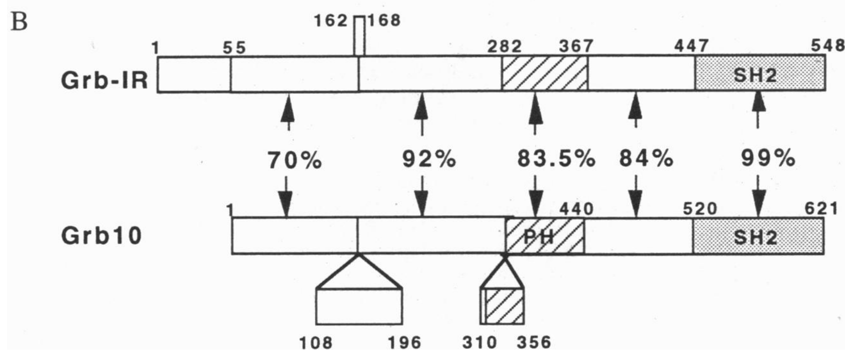

FIG. 1. Deduced amino acid sequence of Grb-IR (*A*) and schematic diagram comparing the domain structure of human Grb-IR and mouse Grb10 (*B*) (the mouse sequence was from ref. 15). Percent sequence identities for the different regions are shown. PH, pleckstrin homology domain.

One of the positive clones (pGAD-IR5) from the HeLa library was shown to completely depend on the IR fusion protein by retransforming the recovered plasmids into yeast containing the bait construct. This clone, which had an insert of 0.9 kb, encodes a 18-kDa polypeptide that is in-frame with the Gal4 activation domain (residues 369–548 in Fig. 1). At the 3′ end of the DNA sequence, there are several stop codons, a putative polyadenylylation site, and a cluster of poly(A).

We then used this 0.9-kb insert to probe various human mRNAs. Northern blot hybridization revealed that expression of this gene was highest in skeletal muscle and pancreas (Fig. 2). In skeletal muscle, there are three mRNA species of approximately 2.2 kb, 5 kb, and 6.5 kb. This size heterogeneity may arise from the utilization of alternative polyadenylylation sites or differential splicing (see below).

To isolate a full-length cDNA clone, we screened a human skeletal muscle library with the 0.9-kb insert. Five overlapping clones with inserts ranging from ≈1.3 to ≈2.3 kb were isolated and characterized. The longest clone contained a 2273-bp insert, a size in close agreement with the observed 2.2-kb mRNA, and has an open reading frame encoding a polypeptide of 548 aa residues with a calculated molecular weight of 62,039 (Fig. 1*A*). There are several in-frame stop codons before the first ATG codon and the sequence surrounding this initiation codon is in good agreement with the consensus Kozak sequence for initiation of translation (12). In addition, the cDNA has a 394-bp 3′ untranslated sequence containing the consensus polyadenylylation signal and a poly(A) tail. A search of several data bases revealed that the cDNA encoded a SH2-domain-containing protein and was given the name Grb-IR. The C-terminal residues 448–548 of Grb-IR contain the putative SH2 domain and this sequence is most closely related (exhibiting 68% identity) to the SH2 domain of Grb7, a protein that binds to the autophosphorylated epidermal growth factor (EGF) receptor and the EGF receptor homolog HER2 (13, 14). However, the sequences of the rest of the two proteins greatly differ and the overall sequence identity between the two proteins is only 38%.

Relationship Between Grb-IR and Grb10. After the completion of the isolation and sequencing of the Grb-IR cDNA, Ooi *et al.* (15) reported the isolation of a cDNA from a mouse library that encodes a SH2-domain-containing protein (called Grb10) that weakly bound to the cytoplasmic tail of the EGF receptor. A comparison of the deduced amino acid sequences of Grb-IR with Grb10 indicates that portions of these two proteins are highly related with a 99% identity in their SH2 domain and 84% identity in the central domain, consistent with Grb-IR being the human homolog of the mouse Grb10 (Fig. 1*B*). However, there are notable difference between these two sequences: (*i*) The N-terminal regions of these two proteins greatly differ, exhibiting differences in a proline-rich region and in the 55 N-terminal residues (Fig. 1*B*). (*ii*) A 46-aa stretch that contains part of the PH domain in Grb10 was absent in Grb-IR (Fig. 1*B*). These differences could be due to alternative splicing of the mRNA encoding these proteins.

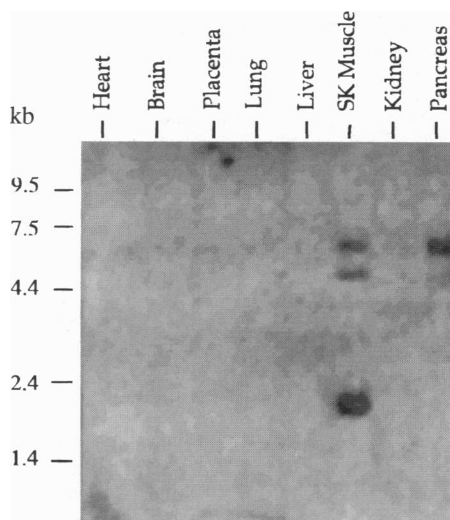

FIG. 2. Tissue expression of Grb-IR mRNA. A human-tissue blot (Clontech) was hybridized to the radiolabeled 0.9-kb cDNA insert derived from pGAG-IR5.

To test this possibility, reverse transcription-coupled PCR experiments were carried out. When specific primers based on the sequence of Grb-IR were utilized that encompassed the PH domain, two specific PCR products were observed with cDNA from HeLa cells, muscle, and fat (Fig. 3*A*). In muscle and fat cells, the major PCR product had a size of ≈1 kb, which was close to the size expected for a PCR product lacking the PH insert. The larger PCR product had a size of 1.1 kb, the size expected for a cDNA encoding a protein containing the additional amino acids in the PH domain. All digestions of the two PCR products with three restriction enzymes (*Mlu* I, *Stu* I, and *Xmn* I) showed the expected size fragments. These results indicate that two distinct mRNAs are present in HeLa, muscle, and fat cells, one encoding a protein with the 46-aa stretch in the PH domain and one lacking this region.

To examine the differences in the N-terminal end of these two proteins, we utilized two different pairs of PCR primers, one based on the 5′ sequence of Grb-IR and the other based on the divergent 5′ sequence of the mouse Grb10 (a common conserved 3′ oligonucleotide primer was used). Specific PCR products with the expected size of 0.8 kb were observed with the human muscle and fat cDNAs when the 5′ Grb-IR oligonucleotide was used (Fig. 3*B*). HeLa cDNA gave two bands of 0.75 and 0.6 kb (Fig. 3*B*). No product was observed under the conditions of these PCRs when the Grb10 primer was used (possibly due to this sequence being from mouse). When the muscle and fat PCR products were digested with *Pst* I, the expected size fragments were observed. These results are consistent with these tissues containing a mRNA with the 5′ end of Grb-IR.

Characterization of the Binding Specificity of Grb-IR *in Vitro*. To determine whether the putative SH2 domain of Grb-IR interacts directly with autophosphorylated IR, GST–Grb-IRc, a bacterially expressed GST fusion protein containing the C terminus of Grb-IR (aa 369–548), was utilized. Lysates from insulin-treated or nontreated CHO-IR cells were incubated with immobilized GST or GST–Grb-IRc fusion protein or with wheat germ agglutinin (WGA) bound to beads (to precipitate total receptor). Bound proteins were detected by antibodies against Tyr(P) or against the β subunit of the IR (Fig. 4*A*). Tyrosine-phosphorylated IR bound efficiently and specifically to GST–Grb-IRc; quantitation of the blots indi-

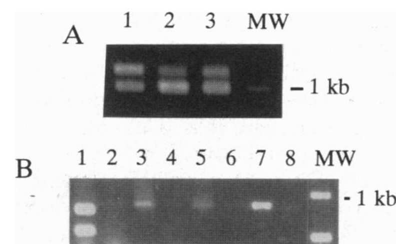

FIG. 3. Grb-IR mRNA heterogeneity in various tissues. (*A*) PCR products amplified from HeLa (lane 1), muscle (lane 2), and fat (lane 3) cDNAs by using primers P4 and P5 flanking the PH domain. (*B*) PCR products amplified from HeLa cell (lanes 1 and 2), muscle (lanes 3 and 4), fat (lanes 5 and 6), or the Grb-IR (lanes 7 and 8) cDNA with primers P1 and P3 (lanes 1, 3, 5, and 7) or P2 and P3 (lanes 2, 4, 6, and 8) from the 5′ end of Grb-IR or Grb10, respectively. Parallel PCRs with only single primers or with both primers and no template gave no reaction product. Lanes MW contain molecular size markers.

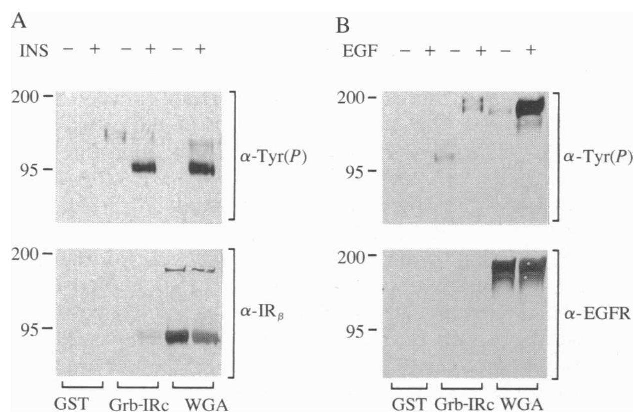

FIG. 4. Association of Grb-IR with the IR and EGF receptor *in vitro*. (*A*) Lysates from insulin (10 nM; INS)-treated and nontreated CHO-IR cells were incubated with GST or GST–Grb-IRc (≈1 μg) bound to GSH-agarose or with WGA-agarose. The agarose-bound proteins were examined by blot analysis with antibodies to Tyr(*P*) (α-Tyr(*P*))/(RC20, Transduction Laboratories, Lexington, KY) or to the β subunit of IR (α-IR$_\beta$) (2H2; gift of Kozui Shii, Harvard University). (*B*) Lysates from EGF (100 ng/ml)-treated or nontreated A431 cells were incubated with GST or GST–Grb-IRc bound to GSH-agarose or with WGA-agarose, and the bound proteins were examined by blot analysis with α-Tyr(*P*) (RC20) or with a polyclonal antibody to EGF receptor (α-EGFR; Upstate Biotechnology, Lake Placid, NY). Molecular masses are shown in kDa.

cated that Grb-IR bound about 80% of the tyrosine-phosphorylated IR and 20% of the total IR.

To test the specificity of the SH2 domain of Grb-IR, we examined whether this protein could bind to the EGF receptor. Lysates from EGF-treated or nontreated A431 cells (a cell with high levels of this receptor) were adsorbed as described and bound proteins were detected with antibodies to Tyr(*P*) or to the EGF receptor (Fig. 4*B*). Less than 10% of the autophosphorylated EGF receptor was detected in the GST–Grb-IRc precipitates, indicating that autophosphorylated EGF receptor can bind to Grb-IRc but much less efficiently than the IR.

Interaction of Grb-IR and IR *in Situ*. To investigate whether Grb-IR associated with IR *in situ*, cell lines expressing a HA-epitope-tagged version of the protein (called CHO-IR/Grb-IR) were selected and characterized. Lysates from the insulin-treated or control cells were adsorbed with antibodies to the IR, the HA epitope, or to Grb-IR and the bound proteins were examined by immunoblot analysis with antibodies to Tyr(*P*) the HA epitope, or IR. Significant amounts of the IR were precipitated with antibodies to the HA epitope from lysates of insulin-treated cells but not of unstimulated cells (Fig. 5*A*). In contrast, the tyrosine-phosphorylated IRS-1, which is present in the lysates in amounts comparable to the IR, was not precipitated with the anti-HA antibodies. Although the antibodies to Grb-IR and the HA epitope precipitated comparable levels of this protein (data not shown), the antibodies to Grb-IR precipitated much less IR. Since the antibodies to Grb-IR were produced against the SH2 domain of this protein, it is likely that these antibodies interfere with this interaction. Low levels of a tyrosine-phosphorylated 66-kDa band, a position consistent with being Grb-IR, were also detected in the precipitates with the antibodies to Grb-IR and the HA epitope. Coimmunoprecipitation of Grb-IR with the autophosphorylated IR can also be observed when the lysates from the insulin-treated cells are precipitated with antibodies to IR (Fig. 5*B*). Quantitation of these blots indicated that about 75% of the total Grb-IR was precipitated with anti-IR antibodies.

Prior studies have indicated that insulin stimulates the tyrosine phosphorylation of two 60-kDa proteins, one that binds to the PI 3-kinase and the other binds to the GTPase-activating protein (GAP) of Ras (16). To test whether Grb-IR could be one of these two proteins, lysates of insulin-treated and control CHO-IR/Grb-IR cells were immunoprecipitated with antibodies to the 85-kDa subunit of the PI 3-kinase or antibodies to GAP. Neither of these precipitates contained detectable amounts of the HA-tagged Grb-IR (Fig. 5*B*). Also, immunoprecipitates of Grb-IR did not contain any PI 3-kinase activity (data not shown).

Effect of Grb-IR Overexpression on IR Tyrosine Phosphorylation of Endogenous Substrates. To test whether the expression of Grb-IR affected insulin signaling, insulin-treated CHO-IR/Grb-IR and CHO-IR cells were compared for their insulin-stimulated increase in tyrosine phosphorylation of endogenous substrates by immunoblot analysis of lysates of both cell types with anti-Tyr(*P*) antibodies. Insulin treatment stimulated an increase in the tyrosine phosphorylation of the IR β subunits in the two cell types to the same degree (Fig. 6*A*). In contrast, the insulin-stimulated increase in the tyrosine phosphorylation of the p60 GAP-associated protein (16) was greatly inhibited in the cells overexpressing Grb-IR and the increase in tyrosine phosphorylation of IRS-1 was partially inhibited. Since tyrosine-phosphorylated IRS-1 is rapidly bound by PI 3-kinase (3), we also measured the insulin-stimulated increase in anti-

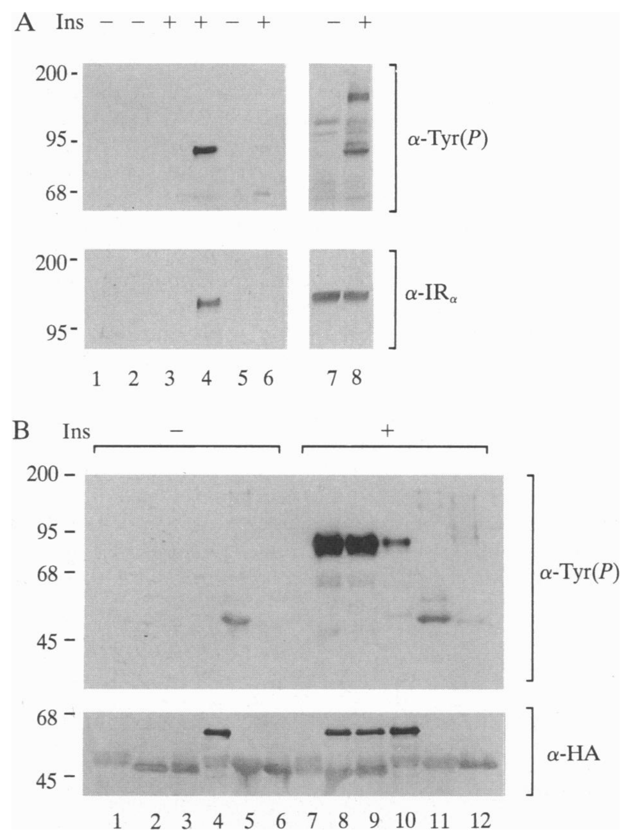

FIG. 5. Association of Grb-IR with IR *in situ*. Lysates (*A*, lanes 7 and 8) or precipitates (*A*, lanes 1–6, and *B*, lanes 1–12) from insulin (Ins)-treated or control CHO-IR/Grb-IR cells were examined by immunoblot analysis with an anti-Tyr(*P*) antibody [α-Tyr(*P*)] (RC20), a polyclonal antibody to the HA tag (α-HA) (Berkeley Antibody), or the monoclonal anti-IR receptor antibody (α-IRα) (3B11; a gift of Kozui Shii) as indicated. Precipitations were with normal mouse immunoglobin (*A*, lanes 1 and 3, and *B*, lanes 1 and 7), a monoclonal antibody to the HA epitope (Boehringer Mannheim) (*A*, lanes 2 and 4, and *B*, lanes 4 and 10); a polyclonal antibody to the SH2 domain of human Grb-IR (*A*, lanes 5 and 6), a monoclonal antibody to the α (5D9) (*B*, lanes 3 and 9) or β (29B4) (*B*, lanes 2 and 8) chain of the human IR, a monoclonal antibody to GAP (*B*, lanes 5 and 11) (Santa Cruz Biotechnology, Santa Cruz, CA), or a polyclonal antibody to the 85-kDa subunit of PI 3-kinase (Upstate Biotechnology) (*B*, lanes 6 and 12). Molecular masses in kDa are shown.

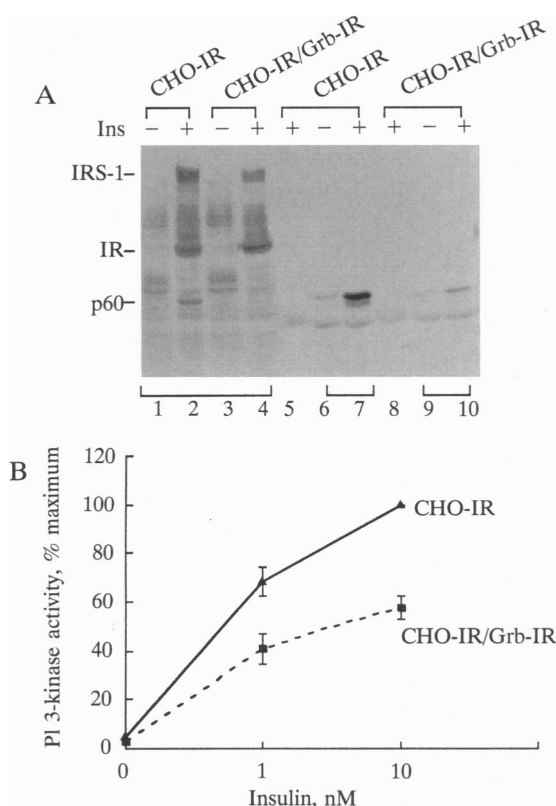

FIG. 6. Effect of Grb-IR expression on insulin-stimulated substrate phosphorylation (*A*) and insulin-stimulated increase in anti-Tyr(*P*)-precipitable PI 3-kinase (*B*). Lysates (lanes 1–4) or precipitates (lanes 5–10) from insulin-treated (+) or control (−) CHO-IR or CHO-IR/Grb-IR cells were analyzed on immunoblots with anti-Tyr(*P*) antibody RC20. Precipitations were with the normal mouse immunoglobin (lanes 5 and 8) or a monoclonal antibody to the GAP-associated p60 protein (lanes 6, 7, 9, and 10) (19). To measure the PI 3-kinase (*B*), the lysates from control or insulin-treated cells were adsorbed with the anti-Tyr(*P*) antibody py20, and the amount of adsorbed PI 3-kinase activity was measured. Results shown are expressed as the percent of activity observed in each experiment, with 10 nM insulin as 100%. Values shown are the mean ± SEM for 6 (at 1 nM insulin) or 12 (at 10 nM insulin) experiments utilizing two clones of CHO-IR/Grb-IR. In each experiment, the amount of protein in the lysates was measured and equivalent amounts of protein from the different lysates were adsorbed.

Tyr(*P*)-precipitable PI 3-kinase activity. Overexpression of Grb-IR in CHO-IR cells was also found to inhibit this response by about 40% (Fig. 6*B*), consistent with the decrease in tyrosine phosphorylation of IRS-1 (Fig. 6*A*).

CONCLUSIONS

In the present study, we have utilized the yeast two-hybrid system (6) to identify a 60-kDa SH2-domain-containing protein (called Grb-IR) that binds with high affinity to the autophosphorylated IR *in vitro* and complexes with the IR *in situ* after insulin treatment of cells. The high level of association of this protein with the autophosphorylated IR (about 75% of the total Grb-IR was found to associate with the autophosphorylated IR) is in contrast to the low levels of association of other SH2-domain-containing proteins with the IR. Moreover, the mRNA for this protein is high in skeletal muscle and present in adipocytes, two insulin-responsive tissues. This protein appears to bind tightly to the autophosphorylated IR but is itself only weakly tyrosine-phosphorylated. These properties distinguish this protein from two other endogenous 60-kDa substrates of the IR tyrosine kinase, both of which are readily tyrosine-phosphorylated and associate with other proteins but not with the IR (16).

Interestingly, overexpression of Grb-IR appears to inhibit the ability of the IR to phosphorylate at least two endogenous substrates, the 60-kDa GAP-associated protein (16) and, to a lesser extent, the IRS-1 protein (3). This latter protein is rapidly bound by the PI 3-kinase after it is tyrosine-phosphorylated and this interaction also appears to be inhibited by the Grb-IR molecule. The activation of PI 3-kinase has been implicated in mediating a variety of biological responses including the stimulation of glucose uptake and the activation of various Ser/Thr kinases (17, 18). Thus, it is possible that increased levels of the Grb-IR protein could serve to inhibit IR signaling, for example, in certain cases of insulin resistance (19). Alternatively, it is possible that Grb-IR protein links the IR to a different set of signaling molecules.

The Grb-IR protein may also have different functions depending on which form of the molecule is expressed. Heterogeneity in the Grb-IR mRNA was observed in Northern blot analyses and PCR studies. In addition, three immunoreactive Grb-IR protein bands (with molecular masses of 50, 65, and 68 kDa) were observed in HeLa cells. The Grb10 cDNA isolated by Ooi *et al.* (15) appears to represent one of these alternate forms of the mouse homolog. The deduced sequence of Grb10 differs from that of Grb-IR in part by containing an additional 46-aa stretch in a putative PH domain. PCR analysis indicated that HeLa cells, muscle, and fat appear to contain both forms of this mRNA. Since the PH domain of proteins has been suggested to be involved in protein–protein interactions (20), these two forms of Grb-IR could have different properties. In addition, the N termini of Grb10 and Grb-IR also differ. This heterogeneity further adds to the possibilities for this protein to interact in the insulin signaling cascade.

We thank Dr. G. Crabtree for the pBEX-1 vector, Dr. J. Glenney for the py20, and Dr. Kozui Shii for the monoclonal antibodies to p60 and the Western blot-positive monoclonal antibodies to the IRα, β. This work was supported by National Institutes of Health Grants DK41765 and DK34962.

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