Identification of the Rat Adapter Grb14 as an Inhibitor of Insulin Actions*

Anne Kasus-Jacobi‡§, Dominique Perdereau‡, Colette Auzan‡, Eric Clauser‡, Emmanuel Van Obberghen‡, Franck Mauvais-Jarvis‡, Jean Girard‡, and Anne-Françoise Burnol***

From ‡Endocrinologie Métabolisme et Développement, CNRS, UPR 1524, 9 rue Jules Hetzel, 92190 Meudon, France, and INSERM U145, Avenue de Valombreuse, 06107 Nice, France

The Journal of Biological Chemistry Vol. 273, No. 40, Issue of October 2, pp. 26026–26035, 1998
Printed in U.S.A.

We cloned by interaction with the β-subunit of the insulin receptor the rat variant of the human adapter Grb14 (rGrb14). rGrb14 is specifically expressed in rat insulin-sensitive tissues and in the brain. The binding of rGrb14 to insulin receptors is insulin-dependent in vivo in Chinese hamster ovary (CHO) cells overexpressing both proteins and importantly, in rat liver expressing physiological levels of proteins. However, rGrb14 is not a substrate of the tyrosine kinase of the receptor. In the two-hybrid system, two domains of rGrb14 can mediate the interaction with insulin receptors: the Src homology 2 (SH2) domain and a region between the PH and SH2 domains that we named PIR (for phospho-tyrosine kinase interacting region). In vitro interaction assays using deletion mutants of rGrb14 show that the PIR, but not the SH2 domain, is able to coprecipitate insulin receptors, suggesting that the PIR is the major binding domain of rGrb14. The interaction between rGrb14 and the insulin receptors is almost abolished by mutating tyrosine residue Tyr1150 or Tyr1151 of the receptor. The overexpression of rGrb14 in CHO-IR cells decreases insulin stimulation of both DNA and glycogen synthesis. These effects are accompanied by a decrease in insulin-stimulated tyrosine phosphorylation of IRS-1, but insulin receptor autophosphorylation is unaltered. These findings suggest that rGrb14 could be a new downstream signaling component of the insulin-mediated pathways.

Insulin is the principal hormone controlling energy metabolism, by modulating metabolic pathways in different target tissues. The liver occupies a central position in the regulation of glucose homeostasis by insulin; insulin inhibits hepatic gluconeogenesis and stimulates glycogen and lipid synthesis. On the other hand, insulin stimulates glucose transport and utilization in skeletal muscle and adipose tissue. These actions of insulin are mediated through a membrane-bound receptor. The insulin receptor as bait. To favor the identification of metabolic effects of insulin have been characterized. Shc and IRSs are ubiquitously expressed, not specifically in insulin-sensitive tissues, and they are also phosphorylated after activation of a number of receptors, including receptor tyrosine kinases, cytokine receptors, and G protein-coupled receptors (8, 10–14). It is therefore possible that other proteins, possibly implicated more specifically in insulin signal transduction, might exist. Recently, different groups have reported the cloning of new proteins supposed to be involved in insulin signaling, since they have been identified by two-hybrid screening using the insulin receptor as bait (15–20). All but two (human MAD2 (19) and Stat5 (20, 21)) are spliced variants of the Grb10 protein. Grb10 was originally cloned as a growth factor receptor-binding protein by interaction with the EGF receptor (22). It is a molecular adapter and a member of the recently emerged Grb7 family of proteins, which comprises Grb7, Grb10, and Grb14 (23, 24). Although its precise role remains to be clarified, Grb10 is likely to be implicated in insulin- and insulin-like growth factor-1-induced mitogenesis (16, 25).

To identify new proteins implicated in insulin signal transduction, we performed a two-hybrid screen of a rat liver cDNA library, using the activated cytoplasmic domain of the insulin receptor as bait. To favor the identification of metabolic effectors, the rat used for the construction of the liver cDNA library encoded by the cDNA ends; kb, kilobases; PAGE, polyacrylamide gel electrophoresis; GST, glutathione S-transferase.

*This work was supported by grants from the Fondation de la Recherche Médicale and by Association pour la Recherche sur le Cancer (A.-F. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

†Recipient of a student fellowship from the Ministère de la Recherche et from the Association pour la Recherche sur le Cancer.‡§To whom correspondence should be addressed. Tel. 33-1-45-07-50-72; Fax: 33-1-45-07-50-39; E-mail: burnol@cnrs-bellevue.fr.

**This paper is available on line at http://www.jbc.org

1The abbreviations used are: IRS, insulin receptor substrate; PTB, phosphotyrosine binding; SH2, Src homology 2; PH, pleckstrin homology; Grb, growth factor-binding protein; rGrb14, rat Grb14; EGF, epidermal growth factor; CHO, Chinese hamster ovary; IR, insulin receptor; PCR, polymerase chain reaction; 5′-RACE, 5′-rapid amplification of cDNA ends; kb, kilobases; PAGE, polyacrylamide gel electrophoresis; GST, glutathione S-transferase.
had been starved for 48 h and refed for 10 h with a diet designed to stimulate transcription of genes implicated in insulin-regulated metabolism. We have cloned a protein displaying a high homology with the human Grb14, a member of the Grb7 subfamily of adapters (24). This protein was then called rGrb14. The data presented in this study suggest that rGrb14 is potentially a new effector of the insulin receptor. In addition, we have identified in rGrb14 a region different from the SH2 domain, which is the main binding domain with the Insulin receptor. This region, named PIR (for phosphorylated insulin receptor-interacting region), is homologous to the BPS domain recently described in Grb10 (26).

**EXPERIMENTAL PROCEDURES**

**Materials**—Synthetic defined dropout yeast media lacking the appropriate amino acids were obtained from Bio 101, Inc. (Vista, CA). Oligonucleotides were purchased from the Pasteur Institut (Paris, France) and Life Technologies, Inc. Monoclonal anti-Myc antibody (9E10 clone) was from Boehringer Mannheim. Monoclonal anti-phosphorytosein antibody (pY20), and polyclonal antibodies against insulin receptor β-subunit were from Transduction Laboratories. Anti-LexA and anti-Gal4 activation domain antibodies were from CLONTECH (Palo Alto, CA). Polyclonal antibodies were raised against the C-terminal 17 amino acids of rGrb14 (Neosystem) and purified on protein A-Sepharose before use. All chemicals were from Sigma France, and enzymes were from New England Biolabs (Beverly, MA).

**Plasmid Constructions**—The intracellular domains of the rat insulin receptor and of the human insulin receptor ATP binding site mutant (IR K1018A) were amplified by polymerase chain reaction (PCR) using pFlu polymerase (Stratagene, La Jolla, CA) and inserted in frame at the BamHI site of the pLex9 plasmid (pLex-IR and pLex-IR K1018). Other insulin receptor mutants in pLex9 vector and pACTII-Shc construct were generated as described previously (4, 5). rGrb14 deletion constructs were generated by PCR and inserted at the BamHI site of pACTII and of pGEX3X (Amersham Pharmacia Biotech). The rGrb14 constructs were generated by PCR and inserted at the HI site of pGAL4 plasmid and subjected to protein A-Sepharose before use. All chemicals were from Sigma France, and enzymes were from New England Biolabs (Beverly, MA).

**Plasmid Constructions**—The intracellular domains of the rat insulin receptor and of the human insulin receptor ATP binding site mutant (IR K1018A) were amplified by polymerase chain reaction (PCR) using pFlu polymerase (Stratagene, La Jolla, CA) and inserted in frame at the BamHI site of the pLex9 plasmid (pLex-IR and pLex-IR K1018). Other insulin receptor mutants in pLex9 vector and pACTII-Shc construct were generated as described previously (4, 5). rGrb14 deletion constructs were generated by PCR and inserted at the BamHI site of pACTII and of pGEX3X (Amersham Pharmacia Biotech). The rGrb14 constructs were generated by PCR and inserted at the HI site of pGAL4 plasmid and subjected to protein A-Sepharose before use. All chemicals were from Sigma France, and enzymes were from New England Biolabs (Beverly, MA).

**The Yeast Two-hybrid Screen of the Rat Liver cDNA Library**—The yeast two-hybrid screen was performed in the yeast strain L40 using on one hand pLexIR, which encodes a constitutively activated insulin receptor β-subunit (27), and on the other hand an oligo(dT)-primed cDNA library from rat liver, cloned in fusion with the Gal4 activation domain in the pGAD352X plasmid (gift from M. Cognet-Vasseur, IN-SEMER U129, Paris, France). After transformation by the lithium acetate procedure (28), yeasts were plated on a tryptophan-leucine-histidine-deficient medium. Colonies growing in the absence of histidine (the first reporter gene) were subsequently tested for β-galactosidase activity (second reporter gene). The plasmids of the library producing yeast colonies of a His"/ LacZ-" phenotype were isolated, and the specificity of association of their products with insulin receptors was tested using pLex-lamin as negative control. The cDNA inserts of these positive plasmids were sequenced, using an Applied Biosystems sequencer (Perkin-Elmer).

**5'- RACE and cDNA Cloning**—To determine and clone the 5'-end of the rGrb14 cDNA, the 5'-RACE technique was used on a rat liver Marathon-Ready premade cDNA library (CLONTECH), with the Advantage cDNA PCR kit (CLONTECH) and a primer 5'-GGCGGCACCTGCTACTGCCACG-3' corresponding to the 5'-end sequence determined on library cDNA insert, according to the manufacturer’s recommendations. We obtained a 250-base pair fragment, which was sequenced and corresponded to the 5'-end of the cDNA. Since the largest library plasmid was lacking only 21 nucleotides of coding sequence, a full-length cDNA containing KpnI and BamHI sites at both ends and a Myc epitope at the 3'-end was reconstructed by PCR with the Pfu polymerase using this plasmid as template and the two following oligonucleotides as primers: 5'-CCGCGGTACCCGAGATCTCCCGCTATGACGCGCCCCGCGCGCCGCG-3' and 5'-CCGCGGTACCCGAGATCTCCCGCTATGACGCGCCCCGCGCGCCGCG-3'. The sequence integrity of the full-length rGrb14 cDNA was verified by DNA sequencing.

**β-Galactosidase Assay**—Yeast strains were transformed by the lithium acetate method of Gietz (28). Quantitative analyses of β-galactosidase activity were performed using a solution assay as described previously (29).

**rGrb14 Expression**—Total RNA was purified from rat tissues or the adipocytic cell line 3T3-F442A using the method of Chomczynski and Sacchi (30). Northern blot analysis was performed as described previously (31) using as a probe a 32P-radioabeled 500-base pair XhoI fragment corresponding to the 5'-end of the rGrb14 cDNA. Rat tissues or 3T3-F442A cells were homogenized in a sucrose buffer (250 mm sucrose, 5 mm Tris-HCl, pH 7.5, 1 mm phenethylsulfonlfluoride, 1 mm pepstatin A, 10 μM aprotinin, 10 μg/ml leupeptin). These cell extracts were subjected to SDS-PAGE and immunoblotted with polyclonal anti-rGrb14 antibodies.

**Overexpression of rGrb14 in CHO-IR Cells**—rGrb14 cDNA was inserted into the KpnI site of the pGEC vector (32). Stable expression of rGrb14 was achieved in CHO-IR cell lines after transfection of the pE-rGrb14 plasmid with a plasmid conferring hygromycin resistance by the calcium phosphate procedure. After limiting dilution, pure clones were identified by Northern blot analysis and Western blot analysis using an anti-Myc antibody. We have used the clone 8A9 for CHO-IR/rGrb14 cells. Preliminary experiments have established that endogenous rGrb14 mRNA can be detected by Northern blot in CHO cells using the rat radiolabeled cDNA probe.

**Immunoprecipitation and Western Blot Analysis**—Confluent CHO-IR cells were serum-deprived for 48 h and stimulated or not by insulin (10-7 M) for 10 min at 37 °C. Cells were solubilized at 4 °C, in 20 mM Tris-HCl (pH 7.4) buffer containing 150 mM NaCl, 10 mM EDTA, 1% Triton X-100, 0.1% bovine serum albumin, and a standard mixture of protease inhibitors (Complete, Roche Diagnostics GmbH, Mannheim), in a solution containing phenethylsulfonlfluoride (2 mM), 20 mM NaF, and 20 mM NaVO4. After a 15-min centrifugation at 15,000 x g, the supernatant was incubated overnight at 4 °C with anti-phosphotyrosine, anti-IR, anti-rGrb14, or anti-IRS-1 antibodies in the presence of protein A-Sepharose. The resulting immunoprecipitates were subjected to SDS-PAGE electrophoresis and immunoblotted with the indicated antibodies. The immunoreactive bands were revealed using the ECL detection kit (Amersham Pharmacia Biotech).

**For in vivo studies in rats, animals were starved for 24 h, anesthetized, and injected with saline or insulin via the saphenous vein. After 10 min, the liver proteins were extracted in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 30 mM sodium pyrophosphate, 50 mM NaF, 0.5% Nonidet P-40, 0.2% Triton X-100, 1 mM NaVO4, 1 mM phenethylsulfonlfluoride, 1 μg/ml pepstatin A, 2 μg/ml leupeptin, 5 μg/ml aprotinin) and centrifuged for 10 min at 6000 rpm at 4 °C. Immunoprecipitation and Western blot analysis were performed as described above.

**In Vitro Interaction Studies**—GST fusion proteins were produced as described previously (29). CHO-IR cells were serum-starved for 24 h and stimulated or not stimulated with insulin (10-7 M) for 10 min at 37 °C. The cell lysates (4 x 106 cells) were prepared as described above and incubated overnight at 4 °C with 3 μg of immobilized GST fusion proteins. After extensive washing, bound proteins were eluted by heating in SDS sample buffer, separated by SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with the indicated antibody, and immunoreactive bands were revealed using the ECL detection kit (Amersham Pharmacia Biotech).

**Metabolic and Mitogenic Actions of Insulin in Parental and rGrb14-transfected CHO-IR Cells**—[14C]Glucose incorporation into glycogen and [3H]thymidine incorporation into DNA were measured as described previously (33). Briefly, confluent cells were stimulated with increasing concentrations of insulin for 1 h prior to incubation with 2 μCi of [14C]glucose (Amersham Pharmacia Biotech) for 3 h. After two phosphate-buffered saline washes, cells were lysed with 30% trichloracetic acid, and the radioactive material was dissolved in 1 M HCl, pH 7.4.

**RESULTS**

**Two-hybrid Screen of a Rat Liver cDNA Library with the Insulin Receptor β-Subunit as Bait**—Seven million independent yeast colonies were tested; 104 clones contained plasmids encoding proteins that exhibited a specific interaction with the insulin receptor β-subunit and not with the kinase-inactive insulin receptor mutated in the ATP binding site (IR K1018A) or with unrelated proteins like lamin. After DNA sequencing,
these specific clones were classified into six different groups encoding distinct proteins (Fig. 1). Three of these proteins were already described as interacting with the insulin receptor: p85α, p85β, and Shc p52 (3, 4, 34–39). Other clones encode the C terminus domain of a splice variant of SH2B, an Src homology-2 domain-containing adapter (40, 41). Clones encoding the full-length Grb7 were also found. Grb7 is a molecular adapter, first isolated by interaction with the EGF receptor (42).

Another group corresponds to 14 plasmids containing inserts of the same cDNA, varying in length from 0.9 to 2.0 kb. This cDNA is the subject of the present study. The longest insert encodes a 531-amino acid protein, which lacks its N terminus. The missing sequence was cloned and identified using the 5′-RACE technique. The full-length protein is 538 amino acids long, as shown in Fig. 2A. The first methionine codon was unambiguously identified by its fairly good context for initiating translation (43) and by the presence of an in-frame stop codon 21 nucleotides upstream. This new protein is an adapter, characterized by the succession of various interacting domains: a central PH domain, a C terminus SH2 domain, and a proline-rich region in the N terminus of the protein. GenBank data base searches revealed significant homology of this protein with the Grb7 family of proteins (including Grb14, Grb7, Grb10, Grb-IR, and Grb10-IR/SV1; the last three are spliced variants of the same gene (16, 44)). The percentage of amino acid identity of the different domains of this protein with members of the Grb7 family is schematized on Fig. 2B. Given the high identity with the amino acid sequence of the human Grb14 (see Fig. 2B) and the homology in nucleotide sequence with the human Grb14 (83%), it is likely that this protein is the rat variant of human Grb14. It was therefore named rGrb14.

**rGrb14 Expression in Different Tissues**—The tissue distribution of rGrb14 was studied by Northern and Western blot analysis. The main transcript is approximately 2.5 kb long, and the shorter fragment is noted in insulin-stimulated liver, tyrosine-phosphorylated IRS-1 is also detected in the anti-rGrb14 immunoprecipitate. rGrb14 is not revealed by anti-phosphotyrosine antibodies in CHO-IR/ rGrb14 cells or rat liver lysates immunoprecipitated with anti-rGrb14 antibodies (Fig. 4, B and C). Thus, rGrb14 is not a substrate of the insulin receptor tyrosine kinase.

**The Molecular Association of rGrb14 to the Insulin Receptor Involves the Tyrosine Kinase Regulatory Loop of the Receptor**—In the two-hybrid system, a kinase-inactive insulin receptor mutant (K1018A, mutated in the ATP binding site) was unable to interact with either rGrb14 or Shc, an insulin receptor substrate taken as control (Fig. 5) (27). This underlines the importance of the receptor activation for the interaction between the insulin receptors and rGrb14. To identify the tyrosyl residues of the insulin receptor that are necessary for this association, we measured the interaction between rGrb14 and insulin receptors mutated on different tyrosyl residues (Fig. 5). The insulin receptor mutants investigated were expressed at similar levels in yeast, as verified by Western blot analysis using an anti-LexA antibody (data not shown). The most striking effect observed was the huge decrease in the interaction between rGrb14 and the insulin receptor mutated in the tyrosine kinase regulatory loop, including Tyr1146, Tyr1150, and Tyr1151. Mutation of Tyr1150 or Tyr1151 induced a 95% decrease in binding to rGrb14, whereas the IR Y1146F mutant showed a less marked impairment in its interaction with rGrb14 (50%...
decrease). Insulin receptors containing double mutations at tyrosyl residues of the kinase loop (IR Y1146/1150F and IR Y1150/1151F) did not bind to rGrb14. In order to distinguish between a defective interaction due to the mutation of the binding site(s) and a defective interaction due to a decrease in the tyrosine kinase activity of these mutant proteins, a comparative study of Shc interactions with these mutants was performed. Interactions between Shc and IR Y1150F, Y1151F,
and 3T3-F442A cells (A). Northern and Western blot analysis is shown of rat tissues (B) and 3T3-F442A cells (C and D). Whole cell extracts from rat tissues (B) and 3T3-F442A cells (D) were immuno- blotted with anti-rGrb14 antibodies, preincubated with or without the antigenic peptide as indicated. A and B, tissue codes are as follows: pancreas (Pa); liver (Li); kidney (Ki); spleen (Sp); brain (Br); white adipose tissue (WAT); brown adipose tissue (BAT); small intestine (In); lung (Lu); heart (He); skeletal muscle (Mu). C and D, 3T3-F442A cells at various stages of differentiation. Lane 1, proliferating fibroblasts; lane 2, confluent cells; lanes 3 and 4, differentiated adipocytes.

or Y1150F/Y1151F were not altered, implying that the tyrosine kinase activity of these mutants was not significantly impaired in this system. In contrast, the tyrosine kinase activity of the insulin receptor mutant Y1146F/Y1150F might be altered, since this mutant did not bind to Shc.

Single mutation of Tyr" was not altered interaction with rGrb14. But as expected, this mutation abolished Shc interaction. Deletion of the two C-terminal tyrosyl residues of the mutant insulin receptor, IR Δ1316/1322, modified neither the interaction with rGrb14 nor the interaction with Shc. Thus, the juxtamembrane domain and the C-terminal domain of the insulin receptor were not implicated in the association with rGrb14.

Identification of the rGrb14 Domains Binding to the Insulin Receptors—The shorter rGrb14 insert isolated in the initial library screen encodes the amino acids 358–538 of the protein, including the SH2 domain (see Fig. 1), suggesting that this fragment contains the region interacting with the insulin receptors. To assess the expected role of the SH2 domain of rGrb14 in the interaction, we have measured the binding of this isolated domain to insulin receptors using the two-hybrid system (Fig. 6A). The rGrb14 SH2 domain has a binding activity for insulin receptors. Mutations of the conserved arginyl residue of the FLVRS motif is supposed to alter the phosphotyrosine binding pocket in the SH2 domain, resulting in a complete loss of its binding activity (45). As expected, the R464K mutated SH2 domain of rGrb14 no longer displays binding activity to insulin receptors. These data provide evidence that the SH2 domain is able to mediate, at least in part, the binding of rGrb14 to insulin receptors.

Surprisingly, the full-length rGrb14 mutant, which contains an R464K mutated and therefore inactive SH2 domain, displayed an interaction with insulin receptors that was similar to that of wild type rGrb14, as did the truncated rGrb14 deleted from its SH2 domain (Fig. 6A). Furthermore, these two mutants did not bind to kinase-inactive insulin receptors (results not shown). These data clearly indicated that the SH2 domain of rGrb14 participated in but was not primarily involved in the interaction with the insulin receptor, which required another domain of the rGrb14 protein. rGrb14 deletion mutant constructs revealed that the major insulin receptor binding activity was contained in a domain of 100 amino acids located between the PI and the SH2 domains (amino acids 340–437). As above, we verified that differences in the interaction of the hybrid proteins were not due to differences in the levels of expression in yeast (data not shown). This binding domain of rGrb14 that mediates the interaction with the activated kinase loop of the insulin receptor was then called PIR (for phosphorylated insulin receptor-interacting region). The binding of the rGrb14 PIR to the insulin receptor was abolished by mutation of Tyr" or Tyr" and was 50% decreased by mutation of Tyr" (Table I). Thus, alterations of the insulin receptor-PIR interaction induced by single tyrosine mutation in the activation loop of the receptor perfectly reflected the interactions of full-length rGrb14 with the same mutants (compare Table I and Fig. 5).

The interaction between the different domains of rGrb14 and the activated insulin receptor was further established using GST pull-down assays. CHO-IR cell lysates from control and insulin-stimulated cells were incubated with GST-rGrb14 fusions. Retained proteins were separated by gel electrophoresis and immunodetected using anti-phosphotyrosine antibodies. As shown in Fig. 6B (top part), the activated insulin receptors coprecipitated with rGrb14 PIR and PIR + SH2 domains but not with the SH2 or the ΔPIR/ΔSH2 domains. Similar interactions were revealed using anti-insulin receptor antibodies (Fig. 6B, lower panel), confirming that these interactions do not occur with the unstimulated insulin receptors. This experiment confirms that the rGrb14-PIR binds to the activated insulin receptors. Interestingly, no interaction was detected between the insulin receptors and the SH2 domain of rGrb14, even after longer exposure of the films.

These experiments showed that the PIR played the predominant role for the rGrb14 interaction with insulin receptors. The rGrb14 PIR did not show any sequence similarity with previously described domains implicated in protein-protein interaction. We were unable to show any alignment with either PTB domains or the KRLB domain, a recently defined domain of IRS-2 that is implicated in the interaction with insulin receptors (46). Data base searches demonstrated that the rGrb14 PIR presented homology exclusively with proteins of the Grb7 family. Sequence comparisons of PIR of the different Grb7 proteins revealed that a 43-amino acid-long region was highly conserved (corresponding to amino acids 365–407 of rGrb14), displaying 93, 77, and 73% of identity when Grb14, Grb10, and Grb7 were compared with rGrb14. The insulin receptor binding activity contained in PIR is likely to encompass this conserved domain.

Effect of rGrb14 on Insulin Actions in CHO-IR Cells—In CHO-IR cells, the stimulation by insulin of glucose incorporation into glycogen was decreased in cells overexpressing rGrb14, as demonstrated by the 62% reduction of the maximal insulin effect, without significant alteration of the EC50 (0.15 and 0.44 nM, respectively, in CHO-IR and CHO-IR/rGrb14 cells) (Fig. 7A). Similarly, the effect of insulin on [3H]thymidine incorporation into DNA was decreased for each insulin concentration tested (Fig. 7B). Similar results were obtained using different impure clones of CHO-IR/rGrb14 cells. These data give evidence that overexpression of rGrb14 has an inhibitory effect on both metabolic and mitogenic actions of insulin.
The inhibition of insulin actions in CHO-IR/rGrb14 cells could be linked to the alteration of one of the first steps of insulin signaling, such as insulin receptor autophosphorylation or IRS-1 tyrosine phosphorylation. CHO-IR and CHO-IR/rGrb14 cell lysates were immunoprecipitated with anti-phosphotyrosine antibodies and immunodetected with anti-insulin receptor antibodies (Fig. 8A). After insulin stimulation, a similar amount of activated insulin receptors was detected in the two cell lines, indicating that the overexpression of rGrb14 did not modify insulin receptor autophosphorylation. Control experiments established that similar amounts of insulin receptors were immunoprecipitated in the two cell lines (data not shown). On the other hand, immunoprecipitation of CHO-IR and CHO-IR/rGrb14 cell lysates with anti-IRS-1 antibodies, followed by an immunodetection with anti-phosphotyrosine antibodies, revealed that after insulin stimulation IRS-1 was less phosphorylated in CHO-IR/rGrb14 that in CHO-IR cells (Fig. 8B). Thus, the overexpression of rGrb14 inhibited the insulin stimulation of IRS-1 tyrosyl phosphorylation.

These experiments show that in CHO-IR cells the overexpression of rGrb14 inhibits insulin actions. The decrease in the effects of insulin observed in the presence of rGrb14 is at least partly explained by a decrease in IRS-1 activation, without alteration of insulin receptor autophosphorylation.

**DISCUSSION**

To identify new partners of the insulin receptor, we chose to screen a library constructed from a rat liver, a target tissue of insulin. From this library, we have isolated the main effectors of insulin signal transduction: p85α, p85β, and Shc but not IRSs. IRS-1 and IRS-2 bind to the insulin receptor through their PH and PTB domains situated in the N terminus of the protein (5, 6, 27, 47). The library was constructed using oligo(dT) primers, which implies that clones encoding IRSs should be more that 4 kb long, which is the upper size limit of such a library. This might explain why we did not isolate them in this screen. Two other proteins, Grb7 and the SH2B isoform, are proteins that are likely to be implicated in cell signaling. Grb7 is known to bind to the EGF receptor, the Ret receptor, and the
platelet-derived growth factor receptor (23, 42, 48, 49), but its association with the insulin receptor was not reported yet. SH2B, which was first cloned by interaction with Fc
\( \gamma \)RI, the \( \gamma \)-subunit of the high affinity IgE receptor, is known to be a potential effector of Janus kinase 2 and also of the insulin receptor (40, 41, 50, 51). However, the physiological implication of these two proteins in insulin signal transduction remains to be established.

The last isolated protein, rGrb14, is the focus of the present study. Several lines of evidence indicate that rGrb14 is likely to be an important effector of insulin signaling. The expression of rGrb14 nicely correlates with the insulin sensitivity of rat tissues. Furthermore, in vivo in rat liver, insulin stimulates the binding of rGrb14 to the insulin receptor \( \beta \)-subunit. In the 3T3F-442A adipose cell line, the appearance of rGrb14 is concomitant with the expression of the insulin receptors and IRS-1 (40, 41, 50, 51). However, the physiological implication of these two proteins in insulin signal transduction remains to be established.

The last isolated protein, rGrb14, is the focus of the present study. Several lines of evidence indicate that rGrb14 is likely to be an important effector of insulin signaling. The expression of rGrb14 nicely correlates with the insulin sensitivity of rat tissues. Furthermore, in vivo in rat liver, insulin stimulates the binding of rGrb14 to the insulin receptor \( \beta \)-subunit. In the 3T3F-442A adipose cell line, the appearance of rGrb14 is concomitant with the expression of the insulin receptors and IRS-1 (40, 41, 50, 51). However, the physiological implication of these two proteins in insulin signal transduction remains to be established.

Whereas other effectors such as IRSs and Shc are recruited and phosphorylated on tyrosyl residues by insulin receptors, rGrb14 binds to activated insulin receptors but is not a substrate of the tyrosine kinase. In agreement with this observation, rGrb14 does not contain any tyrosyl residue in a favorable context for phosphorylation by the insulin receptor tyrosine kinase (YXXM motif; see Ref. 54). The absence of phosphotyrosyl residues on rGrb14 implies that, in contrast to IRSs and Shc, it cannot recruit SH2-containing proteins. Signaling pathways initiated after rGrb14 binding to insulin receptors should then be mediated by mechanisms that are different from those described after IRSs and Shc phosphorylation. This contrasts with the tyrosine phosphorylation of Grb7 induced after stimulation by EGF or Ret (48, 55) and of Grb10 after insulin stimulation (15, 44). The succession of conserved domains in rGrb14 and the sequence comparison in data banks show that it is a member of the Grb7 family of adapters, which already contains Grb7, human Grb14, and multiple isoforms of Grb10 (15–18, 22, 24, 42, 56, 57). All of these proteins were cloned by interaction with activated tyrosine kinase receptors and are supposed to act as signaling proteins. The other interacting domains of rGrb14 contain tyrosine kinase receptors and are supposed to act as signaling proteins. The other interacting domains of rGrb14 contain tyrosine kinase receptors and are supposed to act as signaling proteins.

### Table I

| Interaction of the rGrb14 PIR with the insulin receptor mutants |
|---------------------------------------------------------------|
| Quantifications of the indicated interactions were performed in the two-hybrid system as described in Fig. 5. Results are expressed as percentage of the interaction with the wild type insulin receptor (representing 453 units of Miller). Activities obtained with insulin receptor mutants and wild type were compared using the Student's \( t \) test for significance (\( **, p < 0.01; *** , p < 0.001 \)). |
| IR | Y960F | Y1146F | Y1150F | Y1151F |
|----|-------|-------|-------|-------|
| 100±14 | 105±8  | 50±5** | 1.0±0.3*** | 1.0±0.2*** |

*Fig. 6.* rGrb14 domains interacting with the activated insulin receptor. A, interactions between insulin receptor and wild type (WT) or deletion mutants of rGrb14 are quantified in the two-hybrid system, as described in Fig. 5. Results are expressed as the percentage of the interaction measured between the wild type rGrb14 and insulin receptors (representing 580 units of Miller). S.E. were less than 10% of each value. Activities obtained with rGrb14 deletion mutants were compared with that of wild type rGrb14 using Student's \( t \) test for significance (\( **, p < 0.01; *** , p < 0.001 \)). B, in vitro interaction of rGrb14 domains with the insulin receptor. After insulin stimulation (10\(^{-7}\) M for 10 min), CHO-IR cells were lysed. Proteins were precipitated with GST alone or GST fusion proteins corresponding to different rGrb14 constructs, as indicated. After SDS-PAGE analysis, bound proteins were immunode
tected with anti-phosphotyrosine antibodies (top part) or anti-IR antibodies (bottom part).

2 A. Kasus-Jacobi, unpublished results.

26032

rGrb14 in Insulin Signaling
protein of 120 kDa, phosphorylated on tyrosine residues, coprecipitates with rGrb14 in CHO-IR cells using the in vitro interaction assays. This association is retained by the SH2 domain. After insulin stimulation, the protein either dissociates or is dephosphorylated (see Fig. 6). The identity of this protein is currently under investigation. The protein-interacting role of the SH2 domain of other members of the Grb7 family has already been demonstrated. For example, the Grb10 SH2 domain binds to several unidentified proteins before and after insulin stimulation (16, 44). In addition, the SH2 domain of Grb7 binds Shc, probably at the Y(V/I)N motif as Grb2 (55).

We have defined on rGrb14 a protein-protein interacting region, the PIR, which specifically binds to the phosphorylated insulin receptor. While this work was in progress, it was reported that the homologous domain of Grb10 displays the same binding activity (26). This domain was called BPS (for between PH and SH2) because of its localization. This suggests that the homologous region of the other members of the Grb7 family should also display a similar binding activity. The relative importance of the PIR and of the SH2 domain in the interaction with insulin receptors might vary among Grb7 proteins. Indeed, a 30-amino acid truncation at the carboxyl terminus of the Grb10 SH2 domain suppressed the binding to insulin receptors (17). In contrast, we have shown that rGrb14 deleted of its entire SH2 domain still displays an interaction with insulin receptors similar to that of the wild type rGrb14. Furthermore, in in vitro interaction experiments, the SH2 domain of Grb10, but not of rGrb14, coprecipitates the activated insulin receptors (Ref. 26 and the present study). Thus, it seems that the SH2 domains of Grb10 and rGrb14 play different functions in the association with the insulin receptors. Similarly, it was reported that the Grb7 SH2 domain binds strongly to the ErbB2 receptor tyrosine kinase, whereas the SH2 domain of human Grb14 does not. The substitution of individual amino acids between these two SH2 domains was shown to switch this binding specificity (59). It will be interesting to study the differences in binding and in site recognition between the PIR and SH2 domains of members of the Grb7 family of proteins. These differences should be important for the specificity of interactions between the various growth factor tyrosine kinase receptors and these adapters.

It is interesting to observe that two domains of rGrb14 and Grb10, PIR (or BPS) and SH2 can mediate binding to insulin receptors. In addition, in Grb10 the presence of both domains is necessary for an inhibition of insulin-like growth factor-1-mediated mitogenesis (26). This can be compared with the implication of two binding domains in the interaction between insulin receptors and IRS-1 (PH and PTB (60, 61)) or IRS-2 (PTB and kinase loop regulatory domain (5, 6, 46)). The mechanism of such a binding mediated by two different domains remains to be elucidated. A simple hypothesis could be that the second binding domain is necessary for stabilizing the interaction or that it could confer high specificity as recently shown for tandem SH2 domains (62).

rGrb14, as well as Grb10, binds to the phosphorylated acti-

Fig. 7. Effect of insulin on glycogen and DNA synthesis in CHO-IR cells overexpressing rGrb14. Empty squares, CHO-IR cells; filled squares, CHO-IR/rGrb14 cells. A, glycogen synthesis. Results are the means ± S.E. of nine experiments. B, DNA synthesis. Results are the means ± S.E. of five experiments. Results obtained with or without overexpression of rGrb14 were compared using the t test for significance (*, p < 0.05; **, p < 0.01; ***, p < 0.001).

Fig. 8. Effect of rGrb14 overexpression on insulin-stimulated protein tyrosine phosphorylation. CHO-IR and CHO-IR/rGrb14 cells were stimulated or not with insulin (10^{-7} M) for 10 min and solubilized. A, cell lysates were immunoprecipitated with anti-phosphotyrosine antibodies, and after SDS-PAGE analysis were immunoblotted with anti-IR antibodies. B, cell lysates were immunoprecipitated with anti-IRS-1 antibodies and immunodetected with anti-phosphotyrosine antibodies (top part). To ensure that similar amounts of IRS-1 were immunoprecipitated, the same blot was stripped and rebotted with the anti-IRS-1 antibodies (bottom part).
immunoblot, IRS-1 displays a stronger signal than the insulin receptors, but this can be attributed to its huge number of phosphorylated tyrosyl residues (65).

Further studies are required to definitely establish that rGrb14 is an inhibitor of insulin signal transduction. Indeed, the overexpression of IRS-1 in CHO-IR cells, a positive mediator of insulin actions, has been reported to inhibit insulin-stimulated mitogenesis in CHO-IR cells (66). This paradoxical effect of IRS-1 was explained by the sequestration of downstream effectors, such as Grb2 (67). Thus, overexpression of an effector, by modifying the relative ratio of cellular proteins, can lead either to an amplification of the signal or to an inhibition due to the sequestration of signaling pathway components.

Information about the functional role of rGrb14 can also be obtained by analyzing the molecular interactions between rGrb14 and the insulin receptors. rGrb14 interacts exclusively with the phosphorylated tyrosine kinase regulatory loop. The activation loop was demonstrated as essential for the regulation of the access to the catalytic site of the insulin receptor tyrosine kinase by crystallographic studies (63, 68). Theoretically, rGrb14 could act in two opposite directions by interacting with this loop; it could either inhibit the tyrosine kinase activity by masking access to the catalytic site, or it could maintain the enzyme in an active conformation by stabilizing the phosphorylated loop. Further studies, including structural cocrysalization, are needed to answer to this question.

Acknowledgments—We acknowledge Mireille Cognet-Vasseur (Paris, France) for the rat liver cDNA library; Anne Vojtek (Seattle, WA) and Steve Elledge (Houston, TX) for yeast plasmids and yeast strain; M. F. White, C. R. Kahn (Boston, MA), and T. Issad (Paris, France) for insulin receptor cDNAs; D. Sawka-Verhelle for insulin receptor mutant constructs; and M. Myers Jr. for anti-IRS-1 antibodies. We thank Emmanu elle Plé-Gautier and Martine Leitzke for Northern blots.

REFERENCES

1. White, M. F., and Kahn, C. R. (1994) J. Biol. Chem. 269, 1–4
2. Waters, S. B., and Pessin, J. E. (1996) Trends Cell Biol. 6, 1–4
3. Gustafson, T. A., He, W., Craparo, A., Schaub, C. D., and O’Neill, T. J. (1995) Mol. Cell. Biol. 15, 2500–2508
4. Tartare-Deckert, S., Sawka-Verhelle, D., Murdaca, J., and Van Obberghen, E. (1995) J. Biol. Chem. 270, 23456–23460
5. Sawka-Verhelle, D., Tartare-Deckert, S., White, M. F., and Van Obberghen, E. (1996) J. Biol. Chem. 271, 5850–5853
6. He, W., Craparo, A., Zhu, Y., O’Neill, T. J., Wang, L.-M., Pierce, J. H., and Gustafson, T. A. (1996) J. Biol. Chem. 271, 11641–11645
7. Myers, M. G., Jr., and White, M. F. (1995) Trends Endocrinol. Metab. 6, 199–215
8. Bonfini, L., Migliaccio, E., Pelcici, G., Lanfrancone, L., and Pelcici, P. (1996) Trends Biochem. Sci. 21, 257–261
9. Bell, G. I., Hurant, C. F., Takeda, J., and Gould, W. G. (1993) J. Biol. Chem. 268, 19161–19164
10. Souza, S. C., Frick, G. P., Yip, R., Lobo, R. B., Tai, L.-R., and Goodman, H. M. (1994) J. Biol. Chem. 269, 30085–30088
11. Argelaguer, L. S., Heu, G. W., Myers, M. G., Jr., Billestrup, N., White, M. F., and Carter-Su, C. (1995) J. Biol. Chem. 270, 14685–14692
12. Platanias, L. C., Uddin, S., Yetter, A., Sun, X. J., and White, M. F. (1996) J. Biol. Chem. 271, 278–282
13. Velloso, L. A., Fulii, F., Sun, X. J., White, M. F., Saad, M. J. A., and Kahn, C. R. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 12490–12495
14. Kowalski-Chauvel, A., Pradayrol, L., Vaysse, N., and Seva, C. (1996) J. Biol. Chem. 271, 26356–26360
15. Liu, F., and Roth, R. A. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 10287–10291
16. O’Neill, T. J., Rose, D. W., Pilla, T., Sotta, K., Olefsky, J. M., and Gustafson, T. A. (1996) J. Biol. Chem. 271, 13947–13954
17. Hansen, H., Svensson, U., Zhu, J., Laviola, L., Giorgino, F., Wolf, G., Smith, R. J., and Riedel, H. (1996) J. Biol. Chem. 271, 8862–8866
18. Laviola, L., Giorgino, F., Chow, J. C., Basquero, J. A., Hansen, H., Osi, J., Zhu, J., Riedel, H., and Incevit, J. J. (1996) J. Clin. Invest. 99, 830–837
19. O’Neill, T. J., Zhu, J., and Gustafson, T. A. (1997) J. Biol. Chem. 272, 10035–10040
20. Chen, J., Sadowki, H. B., Kohanski, R. A., and Wang, L.-H. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 2295–2300
21. Sawka-Verhelle, D., Filloux, C., Tartare-Deckert, S., Mothe, L., and Van Obberghen, E. (1997) Eur. J. Biochem. 247, 407–411
22. Ooi, J., Yajnik, V., Immanuel, D., Gordon, M., Moskow, J. J., Buchberg, A. M., and Margolis, B. (1995) Oncogene 10, 1621–1630
23. Margolis, B. (1994) Prog. Biophys. Mol. Biol. 62, 233–244
24. Daly, R. J., Sanderson, G. M., James, P. W., and Sutherland, R. L. (1996) J. Biol. Chem. 271, 12502–12510
25. Morrione, A., Valentinis, B., Resnicoff, M., Xu, S.-Q., and Baserga, R. (1997) J. Biol. Chem. 272, 26382–26387
26. He, W., Rose, D. W., Olefsky, J. M., and Gustafson, T. A. (1998) J. Biol. Chem. 273, 6860–6867
27. O'Neill, T. J., Craparo, A., and Gustafson, T. A. (1994) Mol. Cell. Biol. 14, 6433–6442
28. Gietz, D., St. Jean, A., Woods, R. A., and Schiestl, R. H. (1992) Nucleic Acids Res. 20, 1425–1426
29. Kasue-Jacobi, A., Perdereau, D., Tartare-Deckert, S., Van Obberghen, E., Girard, J., and Burnol, A.-F. (1997) J. Biol. Chem. 272, 17166–17170
30. Komorowski, P., and Sacchi, N. (1987) Anal. Biochem. 162, 156–159
31. Burnol, A.-F., Leturque, A., Leizeau, M., Postic, C., and Girard, J. (1990) Biochem. J. 270, 277–279
32. Ellis, L., Clauser, E., Morgan, D. O., Edery, M., Roth, R. A., and Rutter, W. J. (1986) Cell 45, 721–732
33. Leconte, I., Auzan, C., Debant, A., Rossi, B., and Clauser, E. (1992) J. Biol. Chem. 267, 17145–17147
34. Levy-Toledano, R., Taouis, M., Blaettler, D. H., Gorden, P., and Taylor, S. I. (1992) J. Biol. Chem. 267, 2659–2667
35. Staubs, P. A., Reichart, D. R., Saltiel, A. R., Milarski, K. L., Maegawa, H., Bernahu, P., Olefsky, J. M., and Steely, B. L. (1994) J. Biol. Chem. 269, 27186–27192
36. Lamothe, B., Bucchini, D., Jami, J. and Joshi, R. L. (1995) FEBS Lett. 373, 51–55
37. Tartare-Deckert, S., Murdaca, J., Sawka-Verhelle, D., Holt, K. H., Pessin, J. E., and Van Obberghen, E. (1996) Endocrinology 137, 1019–1024
38. Kozak, M. (1989) Trends Cell Biol. 10, 609–618
39. Burks, D. J., Pons, S., Towery, H., Smith-Hall, J., Myers, M. G., Jr., Yenush, L. and White, M. F. (1997) J. Biol. Chem. 272, 27716–27721
40. Pandey, A., Liu, X., Dixon, J. E., Di Fiore, P. P., and Dixit, V. M. (1996) J. Biol. Chem. 271, 10607–10610
41. Riedel, H., Wang, J., Hansen, H., and Yousaf, N. (1997) J. Biochem. 122, 1105–1113
42. Margolis, B., Silvennoinen, O., Comoglio, P., Ronnrapunt, C., Skolnik, E., Ullrich, A., and Schlessinger, J. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 8984–8988
43. Kosack, M. (1989) J. Cell Biol. 108, 229–241
44. Frantz, J. D., Giorgetti-Peraldi, S., Ottinger, E. A., and Shoelson, S. E. (1997) J. Biol. Chem. 272, 2659–2667
45. Mayer, B., Jackson, P. K., Van Etten, R. A., and Baltimore, D. (1992) Mol. Cell. Biol. 12, 609–618
46. Sawka-Verhelle, D., Baron, V., Mothe, I., Filloux, C., White, M. F., and Van Obberghen, E. (1997) J. Biol. Chem. 272, 16414–16420
47. Hubbard, S. R., Wei, L., Ellis, L., and Hendrickson, W. A. (1994) Nature 372, 746–754