Grb10 is a member of a recently identified family of adapter proteins that are thought to play a role in receptor tyrosine kinase-mediated signal transduction. We identified and isolated the Grb10 SH2 domain based on its interaction with the intracellular domain of the insulin receptor β-subunit using the yeast two-hybrid system. The interaction was specific for the insulin receptor and the insulin-like growth factor-1 receptor, and it required a catalytically active receptor kinase domain and an intact Grb10 SH2 domain. Glutathione S-transferase fusion proteins containing the Grb10 SH2 domain associated in an insulin-dependent manner with insulin receptors from cell lysates and with purified insulin receptors. Co-precipitation experiments revealed the association of cellular Grb10 with hormone-stimulated insulin receptors in cell extracts. The Grb10 SH2 domain did not bind to an insulin receptor lacking 43 amino acids at the carboxyl terminus, and it exhibited highest affinity for a phosphopeptide containing Tyr(P)-1322. Unlike p85 and Syp, which also bind to Tyr(P)-1322, Grb10 was not found to associate with insulin receptor substrate-1. These results suggest that Grb10 is a novel insulin receptor interactive protein and provide direct evidence for an insulin receptor substrate-1-independent function of the insulin receptor carboxyl terminus in protein binding.

The insulin receptor plays important roles in metabolism and growth regulation of target tissues (1–3). Upon insulin stimulation the receptor becomes autophosphorylated on at least six or seven tyrosine residues located in the juxtamembrane, kinase, and carboxyl-terminal regions of its β-subunit (4–7). Many signals are relayed through the insulin receptor substrate-1 (IRS-1), which is phosphorylated by the receptor on various tyrosine residues (8). Phosphorylation of a tyrosine at position 960 in the juxtamembrane region of the insulin receptor is essential for signaling through IRS-1 (9, 10). Among the proteins that bind to IRS-1 are the p85 subunit of phosphatidylinositol 3-kinase (PI3-kinase), Grb2, the tyrosine phosphatase Syp/SHPTP2, and Nck (2). IRS-1 is required for insulin-mediated mitogenesis (11, 12); however, studies using homozygous IRS-1 knockout mice predict important IRS-1 independent insulin signaling pathways (13, 14). A promising candidate, the structurally closely related protein IRS-2 was recently identified, which has signaling functions similar to IRS-1 (15, 16). The Shc proteins represent additional receptor tyrosine kinase substrates that mediate p21<sup>ras</sup> activation in the mitogenic pathway of insulin action (17). We employed the yeast two-hybrid system to identify signaling mediators for alternative pathways analogous to signals emerging from other receptor tyrosine kinases. Here we describe the identification of Grb10 as an IRS-1-independent interactive protein of the activated insulin receptor, and we define the sites of interaction in the receptor and Grb10.

**MATERIALS AND METHODS**

Antibodies—The antibody against the insulin receptor α-subunit was from Biodesign and against the insulin receptor β-subunit from Transduction Laboratories and American Research Products. Anti-SAL-4 and anti-IRS-1 antibodies were from Upstate Biotechnology, and anti-Tyr(P) antibodies from Transduction Laboratories and Upstate Biotechnology. Antibodies 121 and 122 were produced in rabbits against a GST fusion protein containing the Grb10 SH2 domain (Hazelton Research Products). Horseradish peroxidase-coupled anti-iG antibodies (Sigma) were used for immunoblotting.

**Yeast Two-hybrid System**—A BglI-PstI cDNA fragment encoding the carboxyl-terminal 402 amino acids of the receptor β-subunit (18) was ligated to the EcoRI-PstI sites of pGBT9 (19) using an EcoRI-BglI adaptor (5'-ATTCCGGGAGGACGACACAGAT-3' and 5'-TGGCTGCCCTTTCTGCTCGCG-3'). The mutation IR K1018A was introduced by exchanging a BglI-PstI fragment of the insulin receptor bait with the corresponding fragment of the mutated receptor (6). A SalI-BamHI fragment encoding the intracellular domain of the insulin-like growth factor-1 receptor (20) was inserted into the EcoRI-BamHI sites of pGBT9 with an EcoRI-Scal adaptor (5'-AATTCGGTGAGT-3' and 5'-ACTCACCAG-3'). A XbaI-KpnI cDNA fragment encoding the carboxyl-terminal 642 amino acids of rat IRS-1 (8) was inserted into the SalI site of pGBT9 using SalI-XbaI (5'-TCGACTGGTACCCGTG-3' and 5'-CTAGCCGGTACCAGAAGGCGGCAAGAAACAG-3') and KpnI-SalI (5'-CGACCTTGAGG-3' and 5'-TCGACCTCGATGCCTAC-3'). A BglI-PstI fragment encoding the carboxyl-terminal 358 amino acids of protein kinase C α (21) was ligated to the EcoRI-BglI site of pGBT9 with an EcoRI-BglI adaptor (5'-AATTCGGGGAGGACGACACAGAT-3' and 5'-CCGGCTCCCGG-3'). For the screen, yeast strain Y153 (22) was co-transformed with PI3-kinase, phosphatidylinositol 3-kinase; IRS, insulin receptor; Grb10, growth factor receptor-bound protein-10; pY, phosphotyrosine; GST, glutathione S-transferase; SH2, Src homology-2; IGF-1, insulin-like growth factor-1; PAGE, polyacrylamide gel electrophoresis; CHO, Chinese hamster ovary.
the insulin receptor bait and a 10.5 days postconception mouse embryo cDNA library in plasmid pVP16 (23). The specificity of interaction was tested in mating experiments as described (23).

Construction of Fusion Proteins and Protein Purification—A Grb10 cDNA fragment encoding the carboxyl-terminal 108 amino acids was ligated to the BamHI and EcoRI sites of plasmid pGEX-1X (Pharmacia Biotech Inc.) using the polymerase chain reaction primers 5'-CCGG-GATCCATTCAAGACTGAGCATGG-3' and 5'-GGCGGATTCTTC-TATCTTACGGG-3' and was confirmed by sequence analysis. The expressed GST-Grb10 SH2 domain fusion and control GST protein were purified on a glutathione-agarose column (Pharmacia) and stored at 4°C in 0.1 M ammonium carbonate, 1 mM NaCl, pH 7.4, and incubated with 10 μg of GST-SH2 for 2 h at 22°C in a total volume of 200 μl. After washing the bound proteins were separated by SDS-PAGE and visualized by Coomassie Brilliant Blue staining.

Cell Cultures, Immunoprecipitation, and Immunoblotting—CHO/IR (-10° receptors/cell), CHO/IR (-1-3 x 10° receptors/cell), and NIH 3T3AR cells (-6 x 10° receptors/cell) were cultured as described (26, 27). Prior to experiments, cells were incubated for 16 h without serum and treated with 100 μM insulin for 15–20 min. Culture dishes were washed twice with phosphate-buffered saline, and the cells lysed in 50 mM Hepes, 10% glycerol, 137 mM NaCl, 1 mM MgCl2, 1 mM CaCl2, 2 mM EDTA; 10 mM NaF; 10 μM Na3VO4; 10 mM β-glycerophosphate, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, pH 7.4, with 1% Triton X-100 (CHO) or with 1% Brij-97 (NIH 3T3). Proteins were immunoprecipitated (Pharmacia) in the same buffer. The interaction with purified insulin receptors was assayed by incubation with 100 μg/ml GST-Grb10 SH2 for 1 h, followed by a 1.5-h incubation with glutathione beads in 50 mM Hepes, 150 mM NaCl, 2 mM Na2VO4, 0.1% Triton X-100, pH 7.4, at 4°C (24). After four washes, the precipitates were separated by SDS-PAGE and analyzed by immunoblotting.

RESULTS

Isolation of Insulin Receptor Interactive Proteins with the Yeast Two-hybrid System—A two-hybrid library with cDNAs from CHO cells expressing about 10° receptors/cell was screened. The bait plasmid encoded a fusion protein of the GAL4 DNA-binding domain with the carboxyl-terminal 402 amino acids of the insulin receptor b-subunit (18). This includes the complete intracellular region, which had been shown to be constitutively active when separated from the extracellular domain (24). Approximately 107 cells of Saccharomyces cerevisiae strain Y153 (22) were transformed with bait and library, and 13 colonies that grew on medium selective for HIS3 expression were isolated. Among these, 11 colonies showed expression of the lacZ reporter gene. For 10, growth and color were dependent on the presence of both plasmids. Based on the intensity of color in the plate assay, the colonies could be placed into two groups with six stronger (color development within 15–20 min) and four weaker (color development within 30–45 min) interacting clones. Sequence analysis of the plasmid inserts revealed that they all encoded protein fragments containing Src homology-2 (SH2) domains. Three of the weaker interacting clones carried plasmids with distinct inserts that encoded the SH2-containing region of the recently identified signaling mediator Grb10 (29), specifically the carboxyl-terminal SH2 domain of the regulatory subunit p85 of PI3-kinase (28), and one represented a novel sequence. All of the stronger interacting clones carried plasmids with distinct inserts that encoded the SH2-containing region of the recently identified signaling mediator Grb10 (29), specifically the carboxyl-terminal SH2 domain of the regulatory subunit p85 of PI3-kinase (28), and one represented a novel sequence. The specificity of the Grb10 SH2 Domain Interaction—Bait constructs derived from various proteins were employed to test the specificity of the interaction between the Grb10 fragment and the insulin receptor bait. We found that not only transformants with the insulin receptor but also those that expressed a fusion with the intracellular domain of the IGF-1 receptor showed a phenotype characteristic for protein interaction with the Grb10 protein (Fig. 1). Results with all other control transformants, expressing a variety of unrelated baits, such as fusions with the 642 carboxyl-terminal amino acids of IRS-1, the catalytic domain of the serine/threonine protein kinase C α, or the commonly used test protein lamin C were negative. The interaction between Grb10 and the insulin receptor was dependent on a catalytically active tyrosine kinase. This was demonstrated with an insulin receptor point mutation (K1018A), which rendered the kinase catalytically inactive (6). Immunoblotting experiments with antibodies against the GAL4 DNA-binding domain confirmed expression of identical amounts of normal or mutated insulin receptor bait proteins (not shown). In contrast to the normal receptor, interaction of the K1018A insulin receptor with the Grb10 protein was not detectable (Fig. 1). The isolated Grb10 clones contained an SH2 domain as the only discernible structural feature. We identified a truncated sequence of Grb10 lacking 30 amino acids of the SH2 domain at the carboxyl terminus when we randomly amplified fragments from the two-hybrid screen. This truncation exhibited activities only marginally above the experimental background in interaction experiments with the insulin and the IGF-1 receptor enzyme (Fig. 1). In contrast, amino-terminal variations outside of the SH2 domain in other clones did not affect the strength of association to the receptors (Fig. 1). The interaction was thus found to be specific for the insulin/IGF-1 receptors and to be dependent on an active receptor kinase and the intact SH2 domain.

Interaction of Grb10 SH2 Domain Fusion Proteins with Insulin Receptors from Cell Lysates—CHO cells expressing about 10° human insulin receptors/cell (CHO/IR) (26) were lysed with detergent and incubated with a GST fusion containing a minimal Grb10 SH2 domain. Glutathione-Sepharose beads, which bound equal amounts of GST-Grb10 SH2 or GST (not shown), co-precipitated the insulin receptor in experiments with the GST-Grb10 SH2 fusion but not with control GST protein alone (Fig. 2A). The association between the insulin receptor and the Grb10 SH2 domain was strictly dependent on insulin stimulation of the receptor, and the amounts of co-precipitated receptor correlated with the amount of fusion protein in the interaction assay (Fig. 2A). These results are in agreement with the findings from the two-hybrid experiments and emphasize both the role of an active tyrosine kinase and of the Grb10 SH2 domain.
CHO/IR cells was stimulated with insulin
SH2 Domain—

To demonstrate a direct interaction between

in the interaction with the insulin receptor.

Interaction between Purified Insulin Receptors and the Grb10 SH2 Domain—To demonstrate a direct interaction between Grb10 and the insulin receptor, affinity-purified receptor from CHO/IR cells was stimulated with insulin in vitro and incubated with GST-Grb10 SH2 protein or control GST protein. The insulin receptor co-precipitated with glutathione-Sepharose beads as detected in immunoblots (Fig. 2A). As in the experiments with cell lysates, the interaction was dependent on an activated tyrosine kinase and on the Grb10 SH2 domain, whereas the control GST protein did not associate (Fig. 2B). This suggests that Grb10 binds directly to the insulin receptor without the involvement of an intermediate protein.

Mapping of the Interacting Domain of the Insulin Receptor—Synthetic phosphopeptides, each representing one of the seven major tyrosine autophosphorylation sites (30–32) (see "Materials and Methods" for sequences) were compared for their interaction with the GST-Grb10 SH2 domain. We detected binding to Tyr(P)-1322 with high affinity and at a significantly reduced level to Tyr(P)-1316 (Fig. 3A). These assays were complemented by precipitation experiments with the GST-Grb10 SH2 protein, glutathione-Sepharose beads, and lysates from cells that express about 1–3 × 10⁶ copies of a truncated insulin receptor, IR(ΔCT), per cell. The receptor lacks 43 amino acids of the carboxyl terminus including tyrosine residues 1316 and 1322 (26). In contrast to the normal receptor, the mutant did not associate with the Grb10 SH2 domain independent of insulin stimulation (Fig. 3B). Only a small amount of co-precipitated receptor was detected in immunoblots after prolonged exposure in the detection system, which could potentially reflect low affinity binding to any of the other phosphotyrosines in the kinase or the juxtamembrane domain. Densitometric scans of the immunoblot resulted in pellet to lysate ratios of 0.8 for the normal receptor and not more than 0.05 for the mutant. Taken together with the finding that Grb10 binds to the IGF-1 receptor, which lacks a tyrosine homologous to position Tyr-1316 in the insulin receptor, these experiments suggest the most carboxyl-terminal phosphotyrosine of the receptor β-subunit as the major site of interaction between the insulin receptor and Grb10.

Binding Properties of Grb10—Other proteins that bind to the insulin receptor carboxyl terminus, p85 PI3-kinase and the phosphatase Syp, associate preferentially with IRS-1 (2). When we examined co-precipitation experiments with the GST-Grb10 SH2 fusion protein, we did not observe this association for Grb10. IRS-1 was neither detected in protein precipitates from cell lysates containing wild-type insulin receptors nor in those with the carboxyl-terminal truncation, although the ability of the insulin receptor mutant to bind and activate IRS-1 is not impaired (9, 26, 33), and the pool of Grb10 SH2 fusion protein that is available for potential binding to IRS-1 is undepleted (Fig. 3B).

Interaction of Grb10 with Insulin Receptors in Cultured Cells—Grb10 has been immunologically detected in NIH 3T3 cells and is represented on SDS-polyacrylamide gels by several bands ranging from 65 to 80 kDa (29). We used insulin receptor-overexpressing NIH 3T3/IR cells (27) to determine if Grb10 can be found in a complex with the insulin receptor in intact cells. For this purpose cells were incubated with and without insulin and solubilized, and proteins were immunoprecipitated from the lysates with antibodies directed against either the insulin receptor or the Grb10 SH2 domain. As shown in Fig. 4A, two different antibodies against the Grb10 SH2 domain co-immunoprecipitated the insulin receptor upon insulin stimulation of the cells. In a complementary experiment, two different antibodies against the insulin receptor (one directed against the α-subunit and another against the β-subunit) co-precipitated Grb10. Following SDS-PAGE and immunoblotting, the Grb10 antibody, but not preimmune serum (not shown), recognized in both cases the same 75-kDa band in insulin-stimulated lysates (Fig. 4B). This suggests that, at least in NIH 3T3 cells, predominantly one form of Grb10 is bound to the receptor. It remains to be determined whether other forms of Grb10 are also able to associate. On prolonged exposure of the immunoblot in Fig. 4B (not shown), a band of slightly greater mobility was faintly visible, which showed the same pattern of insulin-dependent association with the receptor. The association of
endogenous Grb10 with an insulin-stimulated insulin receptor in intact cells supports a role for Grb10 in insulin action.

**DISCUSSION**

We used the complete intracellular region of the insulin receptor as a bait to screen a two-hybrid library. We identified one known and two previously unknown interacting proteins, including a novel SH2 domain that is being characterized. All sequences also interact with a comparable IgF-1 receptor bait, which is not unexpected given the close structural and functional relationship between these receptors (3). Several isolated clones encode different fragments of the regulatory subunit p85 of PI3-kinase, most frequently of the β-isomer, all of which comprised the more carboxyl-terminal of the two SH2 domains of p85. The interaction of both SH2 domains of p85 with the insulin receptor has been shown before (28, 34).

The screen retrieved multiple, independently cloned inserts of the third end of the Grb10 coding region. Grb10 had been cloned earlier from 12T1 expression libraries based on an association with a carboxyl-terminal epidermal growth factor receptor fragment, but the interaction appeared rather weak, and a role of Grb10 in epidermal growth factor action remains questionable (29, 35). Recently, the RET receptor has been shown to be a putative binding partner for Grb10 (36). Our results provide several independent lines of evidence that Grb10 is a novel insulin receptor interactive protein, including specific binding not only in the two-hybrid system but also in vitro with a GST-Grb10 SH2 fusion protein and in intact NIH 3T3 cells. The Grb10-insulin receptor interaction depends on an active receptor kinase and the intact SH2 domain. The interaction appears to be direct, based on experiments with purified insulin receptors.

Phosphopeptides of each of the seven autophosphorylation motifs in the insulin receptor β-subunit showed that the highest interaction affinity with the Grb10 SH2 domain occurs through Tyr(P)-1322. Consistent with this result, an insulin receptor mutant lacking the carboxyl-terminal 43 amino acids, including tyrosines 1316 and 1322 (R_{cyt}), no longer binds to the Grb10 SH2 domain. A role for Tyr-1322 as the Grb10 binding site is also consistent with the Grb10 interaction with the IgF-1 receptor, in which Tyr-1322 is conserved but Tyr-1316 has been replaced by phenylalanine (3). Although the structural requirements for the interaction are not known, the SH2 domain belongs to group 1, based on a phenylalanine in position 5 of the structural element βD. The composition of Tyr-1322 conforms to the binding properties predicted for this group (Tyr(P)-hydrophilic-hydrophilic-hydrophobic) (37).

The p85 regulatory subunit of PI3-kinase and 5′p bind through SH2 domains to the same carboxyl-terminal motif in the receptor (38). However, both of these proteins associate preferentially with IRS-1, which questions the physiological relevance of their direct association with the insulin receptor (38-40). We could neither detect interaction of the Grb10 SH2 domain with IRS-1 from cells expressing normal insulin receptors nor from those expressing the carboxyl-terminally truncated IR_{cyt}, although this mutant binds and activates IRS-1 normally (9, 26, 33). At present we cannot rule out the possibility that Grb10 might be capable of associating with the recently identified IRS-2, although this protein is structurally very similar to IRS-1 and binds to the same motif in the insulin receptor as IRS-1 (15).

It appears that Grb10 belongs to a family of related proteins, not unlike the members of the IRS signaling system, which depending on the context and physiological condition of the cell may have both shared and specific functions as signaling mediators and may play a role in insulin action. This is supported by the recent report of an insulin receptor interactive protein, termed Grb-IR, for which a role in insulin action has been suggested (41). Compared with Grb10 a distinct tissue distribution and a smaller size has been reported for Grb-IR, but both proteins have, like IRS-1 and IRS-2, similar domain structures of distinct regions with generally high but varying degrees of amino acid similarities (41).

There is increasing evidence that the insulin receptor carboxyl terminus plays an important role not only in mitogenesis (42) but especially in the metabolic functions of the insulin receptor (43). Since our findings imply a role for Grb10 in insulin signaling that is distinct from that of IRS-1 and IRS-2, the investigation of Grb10 may help to clarify the role of the carboxyl-terminal region in insulin signaling.

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