hGrb10α (previously named Grb-IR) is a Src-homology 2 domain-containing protein that binds with high affinity to the tyrosine-phosphorylated insulin receptor and insulin-like growth factor-1 receptor. At least two isoforms of human Grb10, (hGrb10α and hGrb10β), which differ in the pleckstrin homology (PH) domain and the N-terminal sequence, have previously been identified in insulin target tissues such as human skeletal muscle and fat cells. Here we report the cloning of the third isoform of the hGrb10 family (hGrb10γ) from human skeletal muscle and its localization to human chromosome 7. We have also determined the human chromosome localization of Grb7 to 17q21-q22 and Grb14 to chromosome 2. hGrb10γ contains an intact PH domain and an N-terminal sequence that is present in hGrb10α but absent in hGrb10β. RNase protection assays and Western blot analysis showed that hGrb10α and hGrb10γ are differentially expressed in insulin target cells including skeletal muscle, liver, and adipocyte cells. hGrb10γ is also expressed in Hela cells and various breast cancer cell lines. The protein bound with high affinity to the insulin receptor in cells, and the interaction was dependent on the tyrosine phosphorylation of the receptor. hGrb10γ also underwent insulin-stimulated membrane translocation and serine phosphorylation. hGrb10γ phosphorylation was inhibited by PD98059, a specific inhibitor of mitogen-activated protein kinase kinase, and wortmannin, a specific inhibitor of phosphatidylinositol 3-kinase. Taken together, our data suggest that hGrb10 isoforms are potential downstream signaling components of the insulin receptor tyrosine kinase and that the PH domain may play an important role in the involvement of these isoforms in signal transduction pathways initiated by insulin and other growth factors.

Insulin stimulates and regulates cell growth and metabolism by binding to its receptor on the cell membrane. The binding of insulin to the insulin receptor (IR) results in receptor autophosphorylation and receptor tyrosine kinase activation, followed by tyrosine phosphorylation of various cellular substrates including a 185-kDa protein called the IR substrate 1 or IRS-1 (1, 2). Tyrosine phosphorylation of IRS-1 creates docking sites for multiple downstream signaling molecules with specific sequence motifs. One of these functional motifs found in signaling proteins is the Src homology 2 (SH2) domain. The SH2 domain is a sequence of approximately 100 amino acids that binds with high affinity to phosphotyrosine-containing proteins (3). Another functional domain is the pleckstrin homology (PH) domain that has been suggested to play important roles in protein-protein and protein-lipid interactions (4–6).

Although numerous studies have shown that IRS-1 is critical in IR signal transduction, evidence does exist that other proteins may also be involved to transduce a signal from the IR to downstream targets (2). In a search for signaling molecules involved in IR signaling, we used the yeast two-hybrid system to find proteins that interact directly with the cytoplasmic domain of the human IR (7). We identified an SH2 domain-containing protein (hGrb10α, previously named Grb-IR) that binds with high affinity to the autophosphorylated IR. Sequence comparison of hGrb10α with several recently cloned proteins including mGrb7 (8), mGrb10 (9), and hGrb14 (10) suggests that they belong to a special family. All of these proteins contain an SH2 domain at their C termini and a PH domain in the central regions. The SH2 domain of hGrb10 isoforms is 99, 79, and 69% identical to that of mGrb10, mGrb7, and hGrb14, respectively. The PH domains of these proteins are also highly homologous. Although the N termini of these proteins are less conserved, all of them contain a highly conserved proline-rich sequence (P(S/A)IPNPFPEL) (see Fig. 1A), which has been shown to be the potential binding site for SH3 domain-containing proteins (11). In reverse transcription-PCR experiments we also showed that there are at least two isoforms of hGrb10, one that contains and one that lacks an intact PH domain. Expression of hGrb10α, the isoform lacking an intact PH domain, inhibits insulin-stimulated substrate tyrosine phosphorylation and PI 3-kinase activation in cells (7). The mechanism of inhibition has not been characterized.

To further study the role of Grb10 proteins in signaling, we

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1 The abbreviation used are: IR, insulin receptor; CHO, Chinese hamster ovary; GST, glutathione S-transferase; MAP, mitogen-activated protein; PAGE, polyacrylamide gel electrophoresis; PAP, potato acid phosphatase; PH, pleckstrin homology; PI 3-kinase, phosphatidylinositol 3-kinase; SH2, Src homology 2; STS, sequence-tagged site; PCR, polymerase chain reaction; PAUP, phylogenetic analysis using parsimony; kb, kilobase(s); nt, nucleotide(s).
cloned and characterized the PH domain-containing isoform and named it hGrb10γ. We find that hGrb10 isoforms are differentially expressed in insulin target cells and various human breast cancer cell lines and are phosphorylated differently in response to insulin stimulation. In addition, we have shown that the insulin-stimulated hGrb10γ phosphorylation was blocked by wortmannin, a relative specific inhibitor of PI 3-kinase, and PD98059, an inhibitor of MAP kinase. Our findings suggest that Grb10 isoforms are potential signaling components of pathways mediated by insulin and other growth factors and that the PH domain may play an important role in the function of the protein.

EXPERIMENTAL PROCEDURES

cDNA Cloning, Reverse Transcription-PCR, and RNase Protection Assays—The cDNA encoding hGrb10γ was identified by screening a human muscle cDNA library (Stratagene) using a radiolabeled 0.9-kb hGrb10α cDNA fragment (7) as a probe. The nucleotide sequence of this clone was determined by the dye-deoxy chain termination method using the Sequenase 2.0 sequencing kit (Amersham Corp.). Total RNAs from human breast cancer cell lines MCF-7 and MDA-435A were isolated using the Totally RNA kit from Ambion (Austin, TX), and the first strand cDNAs were synthesized using the Superscript kit from Life Technologies. The first strand cDNA was then used to clone hGrb10α into the pBlueScript II SK+ vector and oriented antisense to the T3 RNA polymerase site. The probe was made from a 260-nt sequence of hGrb10α and a 398-nt sequence of hGrb10β or hGrb10γ mRNA. RNase protection assays were carried out by hybridizing the probe with 20 µg of total RNA isolated from several breast cancer cell lines (MCF-7, ZR-75, T47D, MDA-231, MDA-435A, MDA-453A, and MDA-201, MDA-369, or 201B, MDA-435A) and a prostate cancer cell line (DU145). For 36B4 loading control (13), a 145 PstI-PstI fragment was cloned into pGEM4Z, linearized with EcoRI, and transcribed with T7 RNA polymerase. Single-stranded RNA was digested with RNase A, and samples were separated by 8% urea 6% SDS-PAGE (14). tRNA was run as a positive control.

Chromosome Mapping—The chromosome location of the hGrb10 gene was determined by PCR using hGrb10α-specific primers against the Stanford Human Genome Center G3 radiation hybrid panel (Research Genetics, Inc.). The PCR primers were: 5'-CATGGCAAGGAGTAGAGAAGCC-3', which is in the 5'-untranslated region that is specific for hGrb10α and hGrb10γ. The reverse PCR primer (2P) was: 5'-CAGACCACCGAGGGTCTCGA-3', which is located downstream of the cDNA sequence encoding for the PH domain and is common in all three hGrb10 isoforms (Fig. 1B). These primers amplify fragments with predicted sizes of 1.17 and 1.3 kb for hGrb10α and hGrb10γ, respectively. To determine the expression of Grb10 isoforms in different human breast cancer cells and to assess the relative abundance of the isoform mRNAs, a bifunctional antisense [32P]Pribosyn was synthesized in the presence of [32P]uridine triphosphate using a cDNA template consisting of the corresponding PstI fragment was cloned into pBluescript II SK+ vector and oriented antisense to the T3 RNA polymerase site. The probe protects a 260-nt sequence of hGrb10α and a 398-nt sequence of hGrb10β or hGrb10γ mRNA. RNase protection assays were carried out by hybridizing the probe with 20 µg of total RNA isolated from several breast cancer cell lines (MCF-7, ZR-75, T47D, MDA-231, MDA-435A, MDA-201, MDA-369, and 201B, MDA-435A) and a prostate cancer cell line (DU145). For 36B4 loading control (13), a 145 PstI-PstI fragment was cloned into pGEM4Z, linearized with EcoRI, and transcribed with T7 RNA polymerase. Single-stranded RNA was digested with RNase A, and samples were separated by 8% urea 6% SDS-PAGE (14). tRNA was run as a positive control. RNA from Chinese hamster ovary (CHO) cells stably transfected with hGrb10α (CHO/IR/hGrb10α) and hGrb10γ (CHO/IR/ hGrb10γ) were run as a positive control.

Chromosome Mapping—The chromosome location of the hGrb10 gene was determined by PCR using hGrb10α-specific primers against the Stanford Human Genome Center G3 radiation hybrid panel (Research Genetics, Inc.). The PCR primers were: 5'-CATGGCAAGGAGTAGAGAAGCC-3', which is in the 5'-untranslated region that is specific for hGrb10α and hGrb10γ. The reverse PCR primer (2P) was: 5'-CAGACCACCGAGGGTCTCGA-3', which is located downstream of the cDNA sequence encoding for the PH domain and is common in all three hGrb10 isoforms (Fig. 1B). These primers amplify fragments with predicted sizes of 1.17 and 1.3 kb for hGrb10α and hGrb10γ, respectively. To determine the expression of Grb10 isoforms in different human breast cancer cells and to assess the relative abundance of the isoform mRNAs, a bifunctional antisense [32P]Pribosyn was synthesized in the presence of [32P]uridine triphosphate using a cDNA template consisting of the corresponding PstI fragment was cloned into pBluescript II SK+ vector and oriented antisense to the T3 RNA polymerase site. The probe protects a 260-nt sequence of hGrb10α and a 398-nt sequence of hGrb10β or hGrb10γ mRNA. RNase protection assays were carried out by hybridizing the probe with 20 µg of total RNA isolated from several breast cancer cell lines (MCF-7, ZR-75, T47D, MDA-231, MDA-435A, MDA-201, MDA-369, and 201B, MDA-435A) and a prostate cancer cell line (DU145). For 36B4 loading control (13), a 145 PstI-PstI fragment was cloned into pGEM4Z, linearized with EcoRI, and transcribed with T7 RNA polymerase. Single-stranded RNA was digested with RNase A, and samples were separated by 8% urea 6% SDS-PAGE (14). tRNA was run as a positive control. RNA from Chinese hamster ovary (CHO) cells stably transfected with hGrb10α (CHO/IR/hGrb10α) and hGrb10γ (CHO/IR/ hGrb10γ) were run as a positive control.

Chromosome Mapping—The chromosome location of the hGrb10 gene was determined by PCR using hGrb10α-specific primers against the Stanford Human Genome Center G3 radiation hybrid panel (Research Genetics, Inc.). The PCR primers were: 5'-CATGGCAAGGAGTAGAGAAGCC-3', which is in the 5'-untranslated region that is specific for hGrb10α and hGrb10γ. The reverse PCR primer (2P) was: 5'-CAGACCACCGAGGGTCTCGA-3', which is located downstream of the cDNA sequence encoding for the PH domain and is common in all three hGrb10 isoforms (Fig. 1B). These primers amplify fragments with predicted sizes of 1.17 and 1.3 kb for hGrb10α and hGrb10γ, respectively. To determine the expression of Grb10 isoforms in different human breast cancer cells and to assess the relative abundance of the isoform mRNAs, a bifunctional antisense [32P]Pribosyn was synthesized in the presence of [32P]uridine triphosphate using a cDNA template consisting of the corresponding PstI fragment was cloned into pBluescript II SK+ vector and oriented antisense to the T3 RNA polymerase site. The probe protects a 260-nt sequence of hGrb10α and a 398-nt sequence of hGrb10β or hGrb10γ mRNA. RNase protection assays were carried out by hybridizing the probe with 20 µg of total RNA isolated from several breast cancer cell lines (MCF-7, ZR-75, T47D, MDA-231, MDA-435A, MDA-201, MDA-369, and 201B, MDA-435A) and a prostate cancer cell line (DU145). For 36B4 loading control (13), a 145 PstI-PstI fragment was cloned into pGEM4Z, linearized with EcoRI, and transcribed with T7 RNA polymerase. Single-stranded RNA was digested with RNase A, and samples were separated by 8% urea 6% SDS-PAGE (14). tRNA was run as a positive control. RNA from Chinese hamster ovary (CHO) cells stably transfected with hGrb10α (CHO/IR/hGrb10α) and hGrb10γ (CHO/IR/ hGrb10γ) were run as a positive control.

Phylogenetic Analysis—A multiple alignment of all members of the Grb10, Grb7, and Grb14 protein family was generated using ClustalW (15). The alignment was used in a maximum parsimony analysis using Phylogenetic Analysis Using Parsimony (PAUP) (16). The statistical significance of the resulting trees was evaluated using bootstrap analysis within PAUP.

Cell Lines and Anti-hGrb10 Antibody—CHO cells expressing both the human IR and hGrb10α have been described previously (7). To establish cell lines expressing the IR and hGrb10γ, we subcloned hGrb10γ cDNA into the mammalian expression vector pBEX (17) in frames with encoding a 9-amino acid hemagglutinin-tag (YPYDPDYA) to generate the recombinant plasmid pBEX/hGrb10γ. Transfection of CHO/IR cells with plasmids pBEX/hGrb10γ and...
fluorescein-conjugated goat anti-rabbit IgG for 30 min at room temperature. The localization of hGrb10 was examined using a fluorescent microscope.

**Immunoprecipitation, SDS-PAGE, and Western Blot Analysis**—Co-immunoprecipitation of the IR and hGrb10 isoforms were performed as described previously (7). SDS-PAGE was carried out using 10% (w/v) polyacrylamide gels. After electrophoresis, proteins on the gel were transferred to nitrocellulose membranes for Western blot analysis. The immunoblots were blocked at room temperature with buffer containing 10 mM Tris-HCl, pH 7.5, 154 mM NaCl, 0.1% Tween 20, and 1% (w/v) dry milk and then incubated with antibody against hGrb10. hGrb10 isoforms were detected with alkaline phosphatase-conjugated anti-rabbit IgG secondary antibody and a chromatogenous substrate reaction.

**RESULTS**

**Identification and Cloning of hGrb10 γ**—We have previously shown by reverse transcription-PCR that there are at least two hGrb10 isoforms that differ in the PH domain region (7). To clone the cDNA encoding for the isoform containing an intact PH domain, we screened a human muscle cDNA library using a 0.9-kb hGrb10a cDNA as a probe. The deduced amino acid sequence of clone 2, which had an insert of 1.7 kb, is shown in Fig. 1A. This cDNA sequence is identical to that encoding for hGrb10a, except for a 46-amino acid insert (Fig. 1B). A search of...

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**Fig. 1. Deduced amino acid sequence of hGrb10 γ**. A, the potential SH3 domain binding sequence; the PH and the SH2 domains are italicized, underlined, and boxed, respectively. B, the schematic diagram of the domain structures of human Grb10 isoforms. C, the alignment of the N-terminal amino acid sequences of hGrb10 γ, KIA0207, and hGrb10 δ. D, PCR products amplified from CHO/IR/hGrb10a (lane 1), CHO/IR/hGrb10 γ (lane 2), 293 (lane 3), MCF-7 (lane 4), and MDA435A (lane 5) cells using primers P1 and P2. Molecular masses (M.W.) are shown in kb. Std., DNA molecular mass standards.
PCR product with a molecular mass greater than that of containing an intact PH domain. The detection of an additional cell lines and that the major isoform in these cells is the one suggests that mRNAs of both isoforms were present in these cell lines and that the major isoform in these cells is the one containing an intact PH domain. The detection of an additional PCR product with a molecular mass greater than that of hGrb10 was originally identified, the antibody detected three STS markers that have been mapped to their respective chromosome localization. WI-9366 is 100% identical to hGrb7 and has been mapped to YAC 946 E 2, which has been localized to human chromosome 17q21-q22 near topoisomerase 2. Grb14 matches STS marker WI-11831, which has been mapped to human chromosome 11 at position 8.0 cm (9). This region of mouse chromosome 11 is syntenic with human p13-p11, based upon a survey of murine genes that have been mapped to chromosome 11. BLAST searches of the STS data base using hGrb7 and hGrb14 have also identified STS markers that have been mapped to their respective chromosome localization, WI-9366 is 100% identical to hGrb7 and has been mapped to YAC 946 E 2, which has been localized to human chromosome 17q21-q22 near topoisomerase 2. Grb14 matches STS marker WI-11831, which has been mapped to human chromosome 2 at position 836.45 centimorgans.

Expression of hGrb10—It has been shown that Grb7 and Grb14, the other two members of the Grb7/10/14 gene family, were overexpressed in certain breast cancer cells (9, 10). To investigate whether hGrb10 was also expressed in these cells, several breast cancer cell lines were analyzed by RNase protection assays using a probe that spanned the PH splice domain (Fig. 3A). As a positive control, total mRNA from CHO cells stably transfected with hGrb10a (Fig. 3A, PH) or hGrb10v (Fig. 3A, PH) were also analyzed. The majority of cells show a protected band specific for the PH+ isoform (expected size 398 nt) with the splice-variant hGrb10a (expected size, 260 nt) (Fig. 3A).

Using an anti-hGrb10 polyclonal antibody directed against the C-terminal region of the protein, we also examined hGrb10 protein expression in several breast cancer cell lines. Unlike Grb7, which is expressed in only a limited number of breast cancer cell lines (25), an anti-hGrb10 antibody-reactive protein was detected in all breast cell lines studied (Fig. 3B). In ZR-75 and HS578T cells, multiple bands were detected by the antibody, probably due to a phosphorylation-induced gel mobility shift of the protein (see below). Comparison of the apparent molecular mass of this protein with hGrb10 isoforms expressed in the transfected CHO/IR cells suggests that they are the PH domain-containing isoform hGrb10y. In HeLa cells, from which hGrb10a was originally identified, the antibody detected three major protein bands with a molecular mass of approximately 68, 62, and 50 kDa, respectively (Fig. 3C, lanes 1 and 2). The migration distances of the 68- and 62-kDa proteins in the HeLa cells suggests that these proteins are probably the hGrb10 isoforms with or without the deletion in the PH domain.

To evaluate hGrb10 protein expression in insulin target cells, Western blot experiments were carried out on two human hepatocyte cell lines, 3T3-L1 adipocyte cells and human skeletal muscle cells. In human skeletal muscle cells, the antibody detected hGrb10a and a major 50-kDa protein band with unknown identity (Fig. 3D, lanes 5–7). On the other hand, only hGrb10y was detected in human hepatocyte cells (Fig. 3C, lanes 3 and 4). The 50-kDa anti-hGrb10 immunoreactive protein, which was detected in the HeLa cells (Fig. 3C, lanes 1 and 2) was also detected in the human hepatocyte cells (Fig. 3C, lanes 3 and 4). The antibody detected two major protein bands with a molecular mass of approximately 68, 62, and 50 kDa, respectively (Fig. 3C, lanes 1 and 2). The migration distances of the 68- and 62-kDa proteins in the HeLa cells suggests that these proteins are probably the hGrb10 isoforms with or without the deletion in the PH domain.

Fig. 2. Chromosome location of the human Grb10 gene. The integrated map of chromosome 7 is shown between the markers D7S519 and D7S499 (20). The location of the hGrb10 EST probes are shown. These markers are located in the cytogenetic bands 7p12-p11.1.

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4 Mouse Genome Data Base, Mouse Genome Informatics, The Jackson Laboratory, Bar Harbor, Maine. World Wide Web (URL: http://www.informatics.jax.org/).

5 T. Hudson, unpublished data.
human liver cells (Fig. 3C, lanes 3 and 4), and human skeletal muscle cells (Fig. 3D, lanes 5–7) could either be the degradation product of hGrb10 proteins or another isoform of the product. Because its molecular mass is significantly smaller than that of hGrb10b (the calculated mass of which is approximately 61 kDa), it is unlikely that this protein band is the β isoform of hGrb10. Western blot of cell lysates of the differentiated 3T3-L1 adipose cells revealed several protein bands with molecular masses ranging from 48 to 65 kDa (Fig. 3C, lane 5). These proteins are probably the mouse homologues of the hGrb10 isoforms.

Interaction of hGrb10γ with the IR in Cells—To study the interaction between hGrb10γ and the IR in cells, we established cell lines expressing both the IR and hGrb10γ. Lysates from insulin-treated or nontreated CHO/IR, CHO/IR/hGrb10α, or CHO/IR/hGrb10γ cells were incubated with antibody to the β-subunit of the IR (29B4, a gift from R. A. Roth, Stanford University, Stanford, CA) immobilized to protein G-Sepharose beads. After separation by SDS-PAGE and transfer to a nitrocellulose membrane, the IR and its associated hGrb10 isoforms were detected with antibodies to phosphotyrosine (Fig. 4A) or to hGrb10 (Fig. 4B), respectively. A comparable amount of the autophosphorylated IR was precipitated by the anti-IR antibody from the parent CHO/IR cells and the hGrb10 transfected cells lines (Fig. 4A). Both hGrb10α and hGrb10γ could be coimmunoprecipitated by the anti-IR antibody in cells expressing these proteins (Fig. 4B, lanes 4–6). The interaction between hGrb10 isoforms was dependent on the tyrosine phosphorylation of the receptor, and no hGrb10 isoforms were coimmunoprecipitated in the absence of insulin stimulation (Fig. 4, lanes 2–3).

To further characterize the interaction between the IR and hGrb10γ, we studied the localization of the protein in the presence or absence of insulin treatment. In unstimulated
experiments were carried out. Fig. 6

These data suggest that the growth factor-stimulated Grb10 mobility shift was observed (data not shown). HeLa cells and 3T3-L1 adipose cells, no significant insulin-stimulated growth factor and the fibroblast growth factor in NIH3T3 cells expressing endogenous receptors (9). However, in derived growth factor and the fibroblast growth factor in Grb10 (9). In addition, the Grb10 gel mobility shift was shown). These results are consistent with the recent finding that Grb10 moves from cytosol to membrane fractions in the IR-overexpressing Rat1 fibroblasts after insulin stimulation (11).

hGrb10 Undergoes Insulin-stimulated Serine Phosphorylation—Our preliminary studies showed that insulin stimulation resulted in a gel mobility shift of hGrb10γ, suggesting that multiple forms of this protein, for instance differentially phosphorylated species, may occur. To test this hypothesis, hGrb10 isoforms in cell extracts were subjected to phosphatase treatment. Fig. 6A shows an immunoblot of hGrb10α and hGrb10γ from insulin-stimulated or nonstimulated cells after treatment with PAP in the presence or absence of phosphatase inhibitors. Multiple bands of hGrb10γ, which were upward-shifted after insulin stimulation, were detected in lysates when the PAP treatment was carried out in the presence of phosphatase inhibitors (Fig. 6A, lanes 3 and 4). Treatment of the lysates from insulin-stimulated CHO/IR/hGrb10γ cells with PAP in the absence of phosphatase inhibitors converted the broad hGrb10γ bands to a single band with a mobility similar to that of the protein from cells not treated with insulin (Fig. 6A, lanes 3 and 8). PAP had no effect on the gel mobility of hGrb10α (Fig. 6A, lanes 1, 2, 5, and 6). It should be pointed out that the presence of multiple phosphorylated forms of Grb10 was not only in cells overexpressing the protein but also in cells expressing endogenous Grb10 (9). In addition, the Grb10 gel mobility shift was also stimulated by other growth factors such as the platelet-derived growth factor and the fibroblast growth factor in NIH3T3 cells expressing endogenous receptors (9). However, in HeLa cells and 3T3-L1 adipose cells, no significant insulin-stimulated Grb10 mobility shift was observed (data not shown). These results suggest that the growth factor-stimulated Grb10 phosphorylation may be cell type-dependent.

To further characterize the phosphorylation of hGrb10 isoforms, in vivo phosphorylation and phosphoamino acid analysis experiments were carried out. Fig. 6B shows an autoradiograph of 32P-labeled hGrb10γ precipitated from insulin-treated (+) and nontreated (−) CHO/IR/hGrb10γ cells using the antibody against hGrb10. hGrb10γ was phosphorylated under basal conditions, probably due to the overexpression of the receptor and the protein. Insulin stimulation resulted in a 40% increase in the phosphorylation as quantified by PhosphorImager analysis (average of two independent experiments, Fig. 6B). This seems to be an underestimated number, as control experiments showed that our antibody bound to the hyperphosphorylated protein with a much lower affinity (data not shown). The phosphorylation was also significantly decreased for hGrb10α under similar conditions (data not shown). Phosphoamino acid analysis of hGrb10γ indicates that the protein phosphorylation occurred on serine residues (Fig. 6C). However, it should be mentioned that in some of the experiments, we also observed a small but not reproducible tyrosine phosphorylation of hGrb10 by anti-phosphotyrosine immunoblot (data not shown), suggesting that a transient tyrosine phosphorylation of hGrb10 might occur in cells.

hGrb10γ Is a Common Target for Kinases Existing in Both the MAP Kinase and PI 3-Kinase Signaling Pathways—Several enzymes, including MAP kinase and PI 3-kinase, have been shown to be activated by insulin. To test whether kinases involved in these pathways play roles in the phosphorylation of hGrb10γ, we studied hGrb10γ phosphorylation in the presence or absence of specific inhibitors. Wortmannin is a relatively specific inhibitor for PI 3-kinase, and it inhibits the enzyme at nanomolar concentrations (26). PD98059 binds to the inactive form of MAP kinase (MEK1) and prevents its activation by upstream kinases such as c-Raf (27). Fig. 7 shows an immunoblot of hGrb10γ in the presence or absence of these inhibitors. Three different mobility forms of hGrb10γ termed a, b, and c could be visualized in CHO/IR/hGrb10γ cell extracts (Fig. 7, lane 1). Stimulation of cells with insulin increased hGrb10γ phosphorylation, which was evident by a decrease in intensity of the fastest migrating band c and an increase in intensity of bands b and a (Fig. 7, lanes 1 and 2). Pretreatment of cells with PD98059 inhibited the basal phosphorylation of hGrb10γ (Fig.
after stimulating the cells with insulin (Fig. 7, lanes 1–8) or without insulin (lanes 1, 2, 5, and 6) or CHO/IR/hGrb10γ (lanes 3, 4, 7, and 8) cells were treated with PAP (8 μg) for 20 min at 37 °C in the presence (lanes 1–4) or absence (lanes 5–8) of phosphatase inhibitors. hGrb10α or hGrb10γ were resolved by SDS-PAGE, transferred to a nitrocellulose membrane, and detected with a polyclonal antibody against hGrb10, resolved by SDS-PAGE, blotted to a membrane, and visualized by autoradiography.

DISCUSSION

We have identified the PH domain-containing hGrb10α isoform and named it hGrb10γ. We propose a nomenclature that is consistent with other current gene family nomenclatures (Fig. 8). There are three genes located on different chromosomes in human with Grb7 on human chromosome 17q21-q22, Grb10 on human chromosome 7p12-p11, and Grb14 on human chromosome 2.

Each of these genes has an orthologous gene that has been identified in mouse, with a partial cDNA for mouse Grb14 being identified in this study as Expressed Sequence Tag clone 726559 (GenBankTM accession number AA394102). This partial cDNA encodes a part of the PH domain of the murine and other regions of the murine cDNA.

Since each of these genes has an orthologue in the murine genome, we suggest that we refer to the genes as hGrb7/10/14 or mGrb7/10/14. Within the Grb7 and Grb14 genes, no alternatively spliced cDNAs have been reported, although the potential exists for these differences. Within the Grb10 gene in humans, four different isoforms that potentially encode different proteins have been identified. The first human form is human Grb-IR, which we refer to as hGrb10α. Two reports followed with a hGrb10α isof orm, which has a different amino terminus and a complete PH domain (11, 22), which we refer to as hGrb10β. In this report, we describe a hGrb10α isof orm called Grb10γ with a complete PH domain and the same amino terminus observed in hGrb10α. Another isof orm derived from a randomly cloned cDNA (clone KIA0207, GenBankTM accession number D86962) has a third alternative amino terminus and a complete PH domain and is termed hGrb10γ. The mouse Grb10α protein originally identified by screening a NIH3T3 cell cDNA expression library using a radiolabeled tyrosine-phosphorylated epidermal growth factor receptor contains an 80-amino acid region that is not observed in human cDNA clones (9). An isoform of this protein has been reported very recently that lacks the first 25 amino acids of this 80-amino acid insert (28).

The nomenclature for isoforms across species is a difficult issue in general but even more difficult for the Grb10 isoforms. The murine isoform differs in two substantial ways from the human isoforms. 1) It contains an amino terminus different from any of the four hGrb10 isoforms and 2) it contains a region of 55 or 80 amino acids inserted near the PH domain, which has not been observed in humans. The functional consequences of these differences, if any, have not been determined. For these reasons, at this time we propose a distinct nomenclature for the human and murine protein isoforms. As additional murine and human isoforms are reported and the functional consequences of these splicing variations are determined, a consistent nomenclature across species may be developed.

The data presented in this paper have shown that both hGrb10α and hGrb10γ bind with high affinity to the IR in mammalian cells, and the interaction is dependent on receptor tyrosine phosphorylation (Fig. 4). In addition, both isoforms undergo insulin-stimulated translocation from the cytosol to the plasma membrane (Fig. 5 and data not shown). These results suggest that hGrb10α and hGrb10γ are potential signaling molecules in the IR signaling process. However, there are also notable differences between these two isoforms. First, hGrb10γ contains a 46-amino acid sequence contained in the PH domain, which is absent in hGrb10α. The difference in this
Cloning and Characterization of hGrb10γ

![Fig. 8. Classification of Grb10 isoforms.](image)

Functional domain may thus suggest a different role for hGrb10 isoforms in receptor tyrosine kinase signaling. The natural occurrence of two isoforms of hGrb10 that differ in the PH domain may thus provide a mechanism for the regulation of insulin signaling.

The second difference between hGrb10α and hGrb10γ is their cell expression. We have previously shown that although both the ΔPH and PH+ isoforms of hGrb10 mRNAs are expressed in insulin target cells such as skeletal muscle and fat cells, the ΔPH isoform mRNA is more abundantly expressed than that of the PH+ isoform (7). In agreement with these studies, our present Western blot studies reveal that the ΔPH isoform hGrb10α protein is expressed in human skeletal muscle cells (Fig. 3D). The failure to detect hGrb10γ is probably due to a lower expression of this isoform in the cells. On the other hand, the major isoform in human breast cancer cell lines and human liver cells is the PH domain-containing isoform hGrb10γ (Fig. 3, A–C). These data suggest that hGrb10 isoforms vary among different cells or tissues. The different expression of these isoforms could reflect the involvement of these proteins in specific signal transduction or regulation processes, which could depend on functional differences between these isoforms. The observation that only hGrb10γ, but not hGrb10α, undergoes insulin-stimulated serine phosphorylation provides additional evidence that the two isoforms may function differently in the signaling process. Since protein phosphorylation has been shown to be involved in a variety of biological events such as signal transduction and regulation, the phosphorylation of hGrb10γ may be a mechanism that regulates the protein activity in the signaling pathway. For example, phosphorylation may affect the binding of hGrb10 to the IR or to other interacting proteins to amplify or regulate the IR signal. The observation that hGrb10γ phosphorylation is stimulated by insulin (Fig. 6) also suggests a potential role for the protein as a component in IR signal transduction. Another possible role for hGrb10 isoforms in the signaling process may be to provide a scaffolding function. As these isoforms contain several functional domains including the SH2 domain, the PH domain, and an N-terminal proline-rich sequence (PA/S)IPNPFFPEL, which is conserved among several proteins including Grb7 (8), Grb10 (9), and hGrb14 (10), they are capable of binding multiple protein molecules important for the signal transduction process. Recent studies from several laboratories have shown Grb10 binds not only to the insulin receptor but also to other receptors such as the insulin-like growth factor 1R (22, 29, 30), the ELK receptor (31), and Ret (32). These data suggest that the protein may have a more general role in receptor tyrosine kinase signal transduction and regulation.

The inhibition of hGrb10γ phosphorylation by wortmannin and PD98059 suggests that the protein is a potential target of kinase(s) in both the MAP kinase and PI 3-kinase pathways. Whether these enzymes or enzymes downstream of these proteins catalyze the phosphorylation is currently unknown. Sequence analysis of hGrb10 isoforms reveals that the proteins contain three potential MAP kinase phosphorylation sites PXS/T/P (33), suggesting that MAP kinase may directly phosphorylate the protein. It is also interesting to note that insulin can partially overcome the inhibition of hGrb10γ phosphorylation caused by PD98059 but not that caused by wortmannin. These data suggest that there may be multiple phosphorylation sites on hGrb10γ that are targets of different kinases. Identification and characterization of these phosphorylation sites and the hGrb10 kinase(s) should provide further insight into the role of Grb10 in pathways initiated by the IR and possibly other receptor tyrosine kinases.

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Grb7 Protein Family

| Proposed Isoform Name | Accession Number | Amino Terminus | Phosphatase Homology Domain | Chromosome Location |
|----------------------|-----------------|---------------|---------------------------|-------------------|
| hGrb10α              | U34355          | A             | -                         |                   |
| hGrb10γ              | U66665          | B             | +                         | 7p12–p11          |
| hGrb10γ              | A0F01543        | A             | +                         |                   |
| hGrb10δ              | D89692          | C             | +                         |                   |
| mGrb10               | +75’-75’        | mouse Chr 11  | +                         |                   |
| g7Grb10              | U19996          | L76687        | +                         | 2 836.45 cR       |
| g14Grb10             | AA394102        | +             | +                         |                   |
| nGrb14               | D43772          | 17q21–q22     | +                         |                   |
| mGrb7                | M94456          | +             | +                         |                   |
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