GRB10: MORE THAN A SIMPLE ADAPTOR PROTEIN

Mei A. Lim [1], Heimo Riedel [2], and Feng Liu [1]

[1] *Department of Pharmacology, University of Texas Health Science Center at San Antonio, San Antonio, Texas, 78229, USA,* [2] *Department of Biological Sciences, Wayne State University, Detroit, Michigan, 48202, USA*

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1. ABSTRACT

Grb10 is a member of a superfamily of adaptor proteins that includes Grb7 and Grb14. This family of proteins shares a common overall structure, including an N-terminal region harboring a conserved proline-rich motif, a central Pleckstrin homology (PH) domain, a C-terminal Src homology 2 (SH2) domain, and a conserved region located between the PH and the SH2 domains (BPS). Grb10 directly interacts with a number of mitogenic receptor tyrosine kinases including the insulin (IR) and insulin-like growth factor-I (IGF-IR) receptor. Grb10 binds to the regulatory kinase loop of the insulin receptor (IR) via its SH2 and BPS domains. In addition to receptor tyrosine kinases, Grb10 has also been found to interact with non-receptor tyrosine kinases such as Tec and Bcr-Abl, and other cellular signaling molecules such as Raf-1 and the mitogen activated protein (MAP) kinase kinase, MEK.

Overexpression of Grb10 has been shown to inhibit or stimulate insulin/IGF-I signaling depending on the expression levels of the specific isoforms, specific cell context, and/or physiologic endpoint. Genetic imprinting of Grb10 has been linked to the congenital disease, Silver-Russell syndrome, which is characterized by pre- and post-natal growth deficiency. This data suggests that Grb10 may function during embryogenesis in regulating insulin/IGF-I signaling as these growth factors play important roles during development. A role of Grb10 as a potent growth inhibitor during was implicated when disruption of the mGrb10 gene in mice resulted in overgrowth of mutant embryos and neonates. Grb10 is expressed in the central nervous system of mice and rats, which suggests that this protein may regulate neuronal insulin signaling and energy metabolism, consistent with its reported role in metabolic
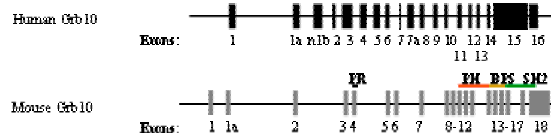
Figure 1. Organization of the mouse and human Grb10 genes. Vertical lines indicate the position of the exons encoding Grb10 transcripts. The exons encoding the PR, PH, BPS and SH2 domains are indicated.
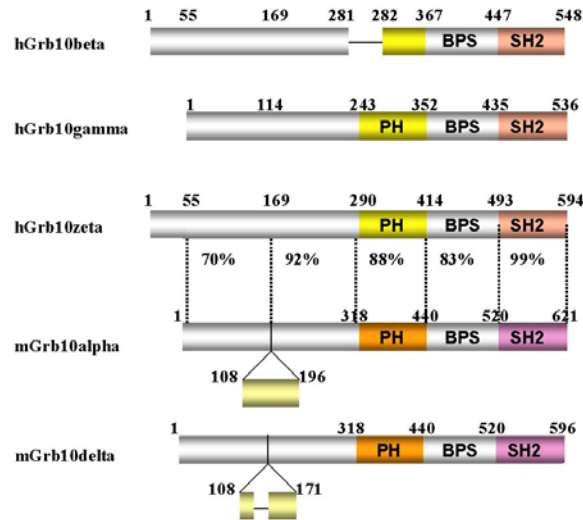

Figure 2. Schematic representation of the different Grb10 isoforms. Note that the scale is different for each protein in order to facilitate homology comparisons of the different domains between the human and mouse splice variants.

insulin action in fat and muscle cells. An important area of future investigation will be to elucidate the mechanism underlying Grb10's ability to regulate peptide hormone action including insulin/IGF-I signaling and to study the physiological role of this adaptor protein in cellular and animal models.

2. INTRODUCTION

Grb10 belongs to the Grb7/10/14 superfamily of adaptor proteins, which is characterized by the presence of an N-terminal proline rich region, a central pleckstrin homology (PH) domain, followed by a domain between the PH and SH2 domains termed the BPS region, and a C-terminal SH2 domain (1-5). The N-termini of the Grb7/10/14 family members exhibits the lowest amino acid homology apart from a highly conserved proline-rich motif (P(S/A)IPNPFPEL). The SH2 domain is the most highly conserved region within the superfamily with the Grb14 SH2 domain exhibiting 67 % and 74 % amino acid identity, respectively, when compared to the corresponding domain of Grb7 and Grb10 (4).

Grb7/10/14 family members also share high sequence homology with the *Caenorhabditis elegans* gene

product Mig-10 (6). Although Mig-10 does not contain a SH2 domain, it possesses both a PH domain and a proline-rich region, reminiscent of the Grb7/10/14 superfamily, leading to the term GM region (for Grb10 and Mig). Mig-10 is involved in the regulation of neuronal cell migration during embryonic development of *C.elegans* (7). In this aspect, Mig-10 is similar in function to the first member of the Grb7/10/14 family, Grb7, which mediates signal transduction regulating cell migration and is implicated in the invasive properties of certain types of cancer (8).

Sequence comparison analysis led to the identification of a conserved region known as the RA-like domain (Ral GDS/AF6 or Ras-Associating) in members of the Grb7/10/14 family of proteins (9). This domain spans approximately 90-100 amino acids, and is located between the proline-rich region and the PH domain. In the case of human Grb7, it encompasses amino acids 101 to 191. Typically, RA domains represent one of several conserved domains that mediate association with members of the Ras superfamily GTPases (10). Whether the putative RA domain in Grb7/10/14 proteins actually promotes binding to Ras GTPases remains to be established.

Despite a growing body of data on the subject, the cellular/physiological role of Grb10 remains unclear, in particular its molecular mechanism of action, partly due to conflicting observations (5). However, a role of Grb10 in regulating the metabolic and mitogenic responses to insulin and IGF-I is well supported by various reports (11-14). In this review, we present the current knowledge on the role of this enigmatic protein in regulating cellular signaling and the potential mechanisms of Grb10 function. We also discuss the link between Grb10 imprinting and Silver-Russell syndrome, and address major unanswered questions regarding Grb10 function.

3. GRB10 DISCOVERY, GENOMIC STRUCTURE AND SPLICE VARIANTS

Grb10 was originally identified as a binding partner of the epidermal growth factor (EGF) receptor, however, a functional role of Grb10 in EGF action has not been established. In this approach, a bacterial expression library derived from NIH3T3 cells was screened with the autophosphorylated carboxyl terminal tail of the epidermal growth factor (EGF) receptor as a probe which identified a cDNA encoding mouse Grb10 (mGrb10) (3). In the same year, the first human variant of Grb10 (hGrb10) was identified as a cellular partner of the insulin receptor in a yeast-2-hybrid screen of a cDNA library derived from HeLa cells using the cytoplasmic domain of the insulin receptor as bait (2). Simultaneously, a similar screen of a mouse embryonic library using the cytoplasmic domain of the murine Ret tyrosine kinase receptor as bait also identified the SH2 domain of mGrb10, an interaction which was duplicated *in vitro* and in 293 T cells (15). In addition, mouse Grb10 was independently identified as an interacting partner of the insulin and IGF-I receptors in a yeast-2-hybrid screen (16). Since then, additional human (4 in total) and mouse (3 in total) Grb10 variants were

discovered and described in different experimental strategies to interact with receptor tyrosine kinases (17-21).

The murine *Grb10* gene resides on the proximal arm of chromosome 11, spans approximately 110 kb, and consists of 18 exons (Figure 1) (3, 22). Mouse Grb10 exon/intron boundaries according to the Ensemble genome server predict that exon 4 encodes the proline-rich (PR) domain, while exons 10-13, 13-16, and 16-18 encode the PH, BPS, and SH2 domains, respectively (22). The mouse *Grb10* gene produces two major transcripts, mGrb10alpha (3) and mGrb10delta (20). While both transcripts initiate at exon 1, mGrb10delta lacks exon 5 (22), resulting in the absence of 25 amino acids located between the proline-rich region and the PH domain (Figure 2) (20). Northern blot analysis with a probe of mostly 3'-untranslated sequences suggested the presence of a third variant of approximately 1.5 kb, termed mGrb10iota. This variant was not recognized by a probe spanning sequences between exons 11 and 16, suggesting the complete absence of the BPS domain (22). However, this transcript was not detected in comparative expression analysis between mouse and human transcripts (23). Currently, all reports on mouse Grb10 (11, 12, 14, 24) appear to be based on expression of the mouse delta variant (20), which is not always properly identified in the published articles due to lack of clarification by the donors of the respective mouse Grb10 cDNAs. Experiments referring to cDNA expression of mouse Grb10 alpha actually represent expression of the mouse delta variant. Potential functional differences between mouse alpha and delta variants remain unknown and could be minor.

The gene encoding human *Grb10* maps to region 7p11.2-p12 of chromosome 7 (18, 25), contains at least 22 exons and encompasses >190 kb of genomic DNA (18). Alternative splicing leads to the generation of multiple isoforms that have been cloned from different human tissues, including hGrb10beta (2), hGrb10gamma (17, 19), and hGrb10zeta (18). The first human variant of Grb10 to be cloned was originally named Grb-IR (2) (insulin receptor-associated growth factor receptor binding protein), and in the current literature, Grb-IR is typically referred to as hGrb10beta, according to the nomenclature proposed by Nantel (21) (for more information about the Grb 7/10/14 superfamily, see http://cbr-rbc.nrc-cnrc.gc.ca/thomaslab/grb7.html). hGrb10beta shows extensive sequence homology to mGrb10alpha, except that it lacked an 88- amino acid insertion near the N-terminus and a 46-amino acid stretch that constituted part of the PH domain (Figure 2). Similarly, hGrb10beta differs from hGrb10zeta (18) (originally called hGrb10gamma) by a 46-amino acid deletion in and around the PH domain, due to loss of exon 7 (Figures 1 and 2). Human Grb10gamma (17, 19) (also called Grb10/IR-SV1 and hGrbIRbeta/hGrb10) varies from hGrb10zeta at the amino acid-terminus because exon 1 is spliced out, resulting in initiation from a different start codon and loss of 55 amino acids from the N-terminus (17, 19). All three human Grb10 isoforms lack the 88 amino acid insertion observed only in mouse Grb10, the functional significance of which remains unresolved. In addition, the presence of five new variants of hGrb10gamma and a novel isoform hGrb10rho have been predicted based on the sequence of the human Grb10 gene (26). The existence of an alternative nomenclature system, which was used prior to the proposal of a unified nomenclature for Grb10 variants, should be noted. In this older system, mGrb10alpha is known as mGrb10, hGrb10beta as hGrb10-IR/alpha and hGrb10zeta as hGrb10gamma.

4. GRB10 TISSUE DISTRIBUTION AND IMPRINTING

4.1. Mouse Grb10

In situ hybridization studies demonstrated that mouse Grb10 mRNA is present during gestation in placenta and most tissues of 13 day old mouse embryos (27). At e14.5, both the Grb10 mRNA and protein are expressed at high levels in a variety of muscle tissues including the face and trunk, intercostal muscles, diaphragm and cardiac muscle, and the limbs (22). High levels of both mRNA and protein were also found in the liver, bronchioles and cartilage of the atlas, ribs and long bones. Grb10 message and protein were also detected in the adrenal gland, pancreatic bud and in the brain of e14.5 embryos. In the adult mouse, northern blot analysis revealed the presence of a single 6 kb message of mGrb10 in heart, kidney, brain, lung, skeletal muscle, and testis ((3) and Hansen and Riedel, unpublished data), whereas expression was consistently absent in spleen and liver. Using a newly generated polyclonal antibody raised against the N-terminus of mGrb10, high levels of mouse Grb10 isoforms were detected in testis, skeletal muscle and brain, and medium levels in the adipose tissue (Ramos, Wang, and Liu, unpublished data). The expression levels of the different isoforms appear to be tissue-specific. The tissue distribution of endogenous Grb10 proteins in insulin-responsive tissues such as skeletal muscle and adipose tissue supports the hypothesis that Grb10 plays an important role in regulating insulin signaling.

Western blot analysis of endogenous and overexpressed mouse Grb10 protein in cell lines such as NIH3T3 and HeLa, respectively, uncovered the presence of multiple protein bands that migrated between 65 and 80 kDa (3). Treatment of mouse Grb10 immunoprecipitates from these cells with potato alkaline phosphatase reduced Grb10 to three forms, indicating that the mouse Grb10 protein is generated by alternate start codons and modified by phosphorylation. Multiple isoforms of endogenous mouse Grb10 was also detected in differentiated 3T3-L1 adipocytes (18).

4.2. Human Grb10

Northern blot analysis revealed that human Grb10 mRNA is transcribed as a 6.5 kb message in a broad range of tissues with the highest level detected in skeletal muscle and pancreas (2, 17, 19). Cardiac muscle and brain expressed relatively high levels of Grb10 transcript, whereas intermediate levels were detected in placenta, lung, liver, kidney, spleen, prostate, testis, ovary, small intestine and colon. Additional transcripts of 5 kb and 2.2 kb were detected in skeletal muscle, possibly products of alternative polyadenylation sites or differential splicing (2, 19).

Expression of the Grb10 protein has been reported in seven different human breast cancer cell lines in comparison to the other two members of the Grb7/10/14 family, which were only found to be expressed in a few select breast cancer cell lines (18). Multiple bands of endogenous Grb10 were also found in human cancer cell lines derived from cervix (HeLaS3, HeLa229), liver (HepG2, Huh7), and in non-cancerous human skeletal muscle cells (18). These multiple forms may represent a combination of post-translationally modified proteins, degradation products and/or Grb10 isoforms. Beyond these observations, the tissue distribution of human Grb10 proteins remains to be elucidated.

4.3 Grb10 imprinting and Silver-Russell Syndrome

In mammals, autosomal genes are typically expressed from both maternal and paternal alleles. However, for a small number of genes termed imprinted genes, the expression is determined by the parent of origin. This genomic imprinting phenomenon, where genes preferentially produce mRNA transcripts from a gene copy derived from the parent of a specific sex, plays important roles in regulating development, growth, and behavior (28-30). Genomic imprinting is caused by gene silencing in the germ line due to methylation of intronic CpG islands (reviewed in (31)). Disruption of imprinting may lead to disease , as expression of a normally silent allele may double the gene dosage, whereas repression of a normally active allele may result in deficiency (32).

The link between Grb10 imprinting and Silver-Russell syndrome (SRS) resulted from the identification of *Meg1/Grb10* as a maternally imprinted gene in mice that is located on proximal chromosome 11, in an area responsible for imprinting effects of pre- and post-natal growth retardation (33, 27). Disruption of the maternal Grb10 allele in mice results in overgrowth of both embryo and placenta, culminating in the birth of mutant mice that are approximately 30 % larger than normal littermates (22). In addition to indicating the role of Grb10 as a potent growth inhibitor, these results demonstrate that changes in Grb10 dosage could, at least in some cases, account for the severe growth retardation characteristic of Silver-Russell syndrome.

In humans, Silver-Russell syndrome (SRS) is characterized by intrauterine and postnatal growth retardation and additional dysmorphic features such as triangular faces, down-turned corners of the mouth, and fifth-finger clinodactyly (34, 35). Approximately 10 % of SRS patients have two maternally derived copies of chromosome 7 (also known as maternal uniparental disomy (mUPD)), (36-38), which represents the highest percentage of mUPD associated with this disease. Two unrelated SRS patients were found to have maternal duplication of the region in chromosome 7 containing genes known to function in the regulation of growth and development, such as *IGFBP1, IGFBP3*, and *Grb10* (39, 40). The roles of *IGFBP1* and *IGFBP2* in the aetiology of SRS were ruled out as these genes exhibit biallelic expression and were not imprinted (41, 42). Taken together, imprinting studies in mice and humans implicate Grb10 as a strong candidate for the maternally imprinted gene associated with the aetiology

of SRS. Reports of the inhibitory effects of Grb10 on some of the molecular interactions downstream of the insulin/IGF-I receptor may suggest a mechanism as to how *Grb10* imprinting could lead to SRS. Inheritance of two maternal copies of *Grb10* may lead to a double gene dosage effect, resulting in pre- and post-natal growth retardation by inhibition of the growth promoting effects of insulin/IGF-I.

Currently, the role of Grb10 as one of the contributing factors of SRS is intensely debated as there are conflicting reports regarding the imprinting status of Grb10. Mouse Grb10 has been reported as a maternally expressed gene in almost all tissues and placentas of 13-day old embryos as well as in heart, kidney, lung, liver, and brain of adult mice (27). This is in contrast to the pattern of expression reported in humans where maternal **repression** of Grb10 was observed specifically in the developing central nervous system including brain and spinal cord, with biallelic expression detected in peripheral tissues (43). This is the first reported incidence of opposite imprinting in human and mouse homologues. In addition, the expression of Grb10 in human tissues is more complex, exhibiting both tissue- and isoform-specific regulation (26). In human fetal brains, most splice variants of Grb10 are transcribed exclusively from the paternal allele. Maternal monoallelic expression of human Grb10 is found in skeletal muscle but is restricted to the Grb10gamma isoform (26). Since SRS is associated with maternal duplication of chromosome 7, the findings of biallelic expression of Grb10 in most fetal tissues and paternal expression in the central nervous system argue against a major role for Grb10 in the aetiology of SRS. The significance of the tissue-specific reciprocal imprinting pattern of Grb10 in brain and muscle remains unknown. Therefore, it remains to be established whether Grb10 plays a role in SRS in humans.

5. STRUCTURE AND FUNCTION OF GRB10 DOMAINS

5.1 Proline rich region

Grb10 splice variants share a proline-rich region at their N-terminus which is also found in Grb7 and Grb14. In addition to containing the motif PS/AIPNPFPEL that is conserved across the Grb7/10/14 family members, Grb10 family members contain either one (PPVLTPGSLPP for human) or two (PPSVAPSSLPP and PPPPSQPP for mouse) additional proline-rich motifs. The second motif present in mouse and human Grb10 isoforms shares 63.6% identity, with prolines being highly conserved.

Sequences that are rich in proline have been reported to act as ligands for protein-interaction domains such as the src-homology domain 3 (SH3) and WW domains (small globular domains rich in tryptophan) (44). The SH3 domain of the non-receptor tyrosine kinase, c-Abl, has been reported to interact with rat Grb10 *in vitro*, and this binding was abolished by incubation with a proline-rich peptide mimetic (SLPAIPNPFPEL) (19). While the SH3 domain of c-Abl associated with the proline-rich region of Grb10 it remains unknown whether this interaction occurs *in vivo* or whether the entire c-Abl associates with Grb10. In a conflicting report, the SH2

domain as opposed to the proline-rich region of Grb10 was reported to interact with Bcr-Abl (45).

Two Grb10 interacting proteins, GIGYF1 (<u>G</u>rb10 <u>I</u>nteracting <u>GYF</u> Protein <u>1</u>) and GIGYF2, containing a GYF domain were identified in a yeast-2-hybrid screen when the N-terminal of mGrb10delta was used as bait (46). Deletion analysis revealed that at least two of the three proline-rich motifs of mGrb10 are required for binding to both GIGYF1 and GIGYF2. IGF-I stimulation increases the affinity of GIGYF1 for mGrb10delta and results in a transient association of GIGYF1 and mGrb10 with the IGF-I receptor. Thus GIGYF1 may promote the association of the IGF-I receptor with mGrb10. Overexpression of the mGrb10 binding region of GIGYF1 enhances IGF-I receptor-stimulated tyrosine autophosphorylation. Therefore, mGrb10 and GIGYFs may cooperate to regulate receptor signaling (46). As the human and mouse Grb10 isoforms only share one identical proline-rich motif, it would be interesting to determine whether the binding affinity for the GIGYF proteins differ between human and mouse isoforms.

Cell membrane permeable peptide mimetics of the N-terminal mouse Grb10 proline-rich sequence indicate a specific role of this motif in insulin-mediated metabolic responses and in insulin- and IGF-I-induced mitogenic responses (12, 14). The molecular role of the Grb10 proline rich region requires further clarification which may benefit from the solution of the crystal structure of Grb10, independently or associated with its partner protein.

5.2. PH domain

The pleckstrin homology (PH) domain is a structural protein module of approximately 100 amino acids originally identified in the cytoskeletal protein pleckstrin (47, 48). This domain is found in diverse proteins involved in cellular signaling, cytoskeletal organization, and regulation of membrane trafficking (49, 50). Although PH domains are also reported to mediate protein-protein interactions, the major role of this domain lies in the binding of phosphoinositides which are typically produced in response to activation of cell surface receptors, resulting in the recruitment of PH domain proteins to the plasma membrane (reviewed in (51)). Unlike the PH domain of Grb7 (52), it is not known whether the PH domain of Grb10 binds to phosphoinositides. However, the PH domain of Grb10 is not essential for the ability of Grb10 to translocate to the plasma membrane as hGrb10beta, which lacks an intact PH domain, still exhibited insulin-stimulated membrane translocation (18). Instead, an intact PH domain enhances the association of Grb10 with the insulin and IGF-I receptors, which is mediated by the BPS and SH2 domains (53). The presence of an intact PH domain may produce a more favorable conformation for Grb10 to interact with the receptors. Along with the SH2 and BPS domain, the PH domain is also involved in mediating the oligomerization of Grb10 in yeast-2-hybrid studies (54). In summary, although the molecular details remain unclear, the PH domain of Grb10 appears to be involved in mediating intramolecular interactions such as oligomerization as well as intermolecular interactions with other proteins.

5.3. SH2 domain

The Src-homology 2 (SH2) domain represents a protein module of approximately 100 amino acids that was originally identified in the retroviral oncogene v-Fps as a region that is not required for tyrosine kinase activity but is involved in regulating the function of the protein via intermolecular and intramolecular interactions (55). SH2 domain containing proteins range from adaptor proteins including the Grb family members, scaffold proteins, kinases, phosphatases, transcription factors to cytoskeletal proteins (reviewed in (56)). SH2 domains bind to phosphotyrosine residues, and specificity of binding is imparted by selective recognition of 3-6 amino acid residues C-terminal to the phosphotyrosine (57, 58). Thus the sequence context around the tyrosine phosphorylation site determines which SH2 domain bearing protein is recruited and hence which biochemical pathway is activated.

The crystal structure of the hGrb10 SH2 domain revealed that unlike typical monomeric SH2 domains, the SH2 domain of hGrb10 is dimeric in solution, and this observation was confirmed by gel filtration and sedimentation experiments (59). The structure of the Grb10 SH2 domain resembles the structure of other SH2 domains in that it consists of a core anti-parallel beta sheet flanked on both sides by an alpha helix. Residues from the C-terminal half of the SH2 domain, primarily in alpha-helix B, contribute to the Grb10 SH2 dimer interface. Sequence alignment reveals that the residues in the Grb10 SH2 dimer interface are strictly conserved among members of the Grb7/10/14 family members except for Phe-496, which is substituted with tyrosine in Grb7 (59). This implies that the SH2 domain of Grb7 and Grb14 may also form dimers in solution. Further confirmation of the dimeric nature of Grb10's SH2 domain was obtained when Phe-515, which is located at a key position at the center of the dimer interface, was mutated to Arg, resulting in the presence of only SH2 domain monomers. Full length Grb10gamma is capable of oligomerization, although whether this protein exists as an elongated dimer or tetramer was not unequivocally established (59). Solution of the crystal structure also yielded information on the binding preference of the SH2 domain of Grb10. In a typical SH2 domain-phosphopeptide association, the phosphotyrosines in the +1 (P+1) and +3 (P+3) positions of the phosphopeptide fit into two deep pockets in the SH2 domain. However, the binding pocket for the P+3 residue in the Grb10 SH2 domain is absent. This suggests that the dimeric Grb10 SH2 domain will favor binding of dimeric, turn-containing phosphotyrosine sequences, such as the activation loop of the two beta subunits of the insulin and insulin-like growth factor-1 receptors. Various signaling mediators have been reported to associate with the SH2 domain of Grb10, including receptor tyrosine kinases for insulin, IGF-I, EGF and PDGF (2, 3, 16, 17, 12).

The Grb10 SH2 domain also interacts with phosphorylated tyrosine residues on the non-tyrosine kinase growth hormone receptor (60). Non-receptor-tyrosine-kinase binding partners include the oncoprotein Bcr-Abl (45) and the tyrosine kinase ELK1 (61). The Grb10 SH2

domain can also associate with proteins in a phosphotyrosine-independent manner as reported for Raf1, MEK1, and Nedd4 (neuronal precursor cell-expressed developmentally down-regulated 4) (21, 62).

5.4. BPS domain

Full length Grb10 interacted more efficiently with the IR *in vitro* than the SH2 domain alone, indicating the presence of an additional interacting domain (19). Mapping studies using the yeast-2-hybrid system and *in vitro* protein interaction assays unveiled the presence of a relatively small domain (approximately 50 amino acids), termed BPS (located between the PH and SH2 domains), which is highly conserved within, and unique to, the Grb7/10/14 family members (63). This domain represents a major interface with the IGF-IR, which shares high sequence homology to the IR but not with the EGF receptor (63). This may help explain the weak association of Grb10 with the EGF receptor (3). Thus far, there is no evidence supporting a functional role for Grb10 in the EGF receptor signaling pathway. A key role of the Grb10 BPS domain in the interaction with the IGF-I receptor and to a lesser degree, with the insulin receptor, is supported by competition experiments (12).

An interaction of the mGrb10 BPS domain with Nedd4 was detected in a yeast-2-hybrid screen of a mouse embryonic library (62). The Nedd4 protein is a ubiquitin protein ligase composed of three WW domains and a hect (homologous to the E6-AP carboxyl-terminus) domain bearing homology to a ubiquitin ligase (64, 65). Although the BPS domain can interact independently with Nedd4 in the yeast-2-hybrid system, the stronger interaction is mediated by the SH2 domain (62). The Grb10/Nedd4 complex regulates ubiquitination and stability of the IGF-I receptor (24).

The molecular mechanism of the BPS domain interaction with its partners remains largely uncharacterized. Mutation of the paired tyrosines Y1162/1163 in the IR activation loop to phenylalanine reduced binding of the IR by the individual BPS and SH2 domains, indicating that these tyrosine residues are essential for the interaction of both domains (63). It remains to be clarified whether the BPS domain interacts directly with the activation loop tyrosines or whether autophosphorylation of these tyrosines causes a conformational change within the kinase domain, exposing a binding site for the BPS domain. The former possibility was ruled out as interaction of the combined BPS and SH2 domains with the phosphorylated kinase domain of the IR was not disrupted in a peptide competition assay using a phosphorylated peptide containing all three autophosphorylation sites (Tyr1158/ 1162/ 1163) of the IR as the competitor (66). Whether the Grb10 BPS domain or the SH2 domain constitutes the main interface in the association with the insulin receptor remains unresolved.

6. THE ROLE OF GRB10 IN SIGNALING

A large number of signaling mediators ranging from receptor tyrosine kinases, cytoplasmic serine/threonine kinases to signaling adaptors have been reported to interact with various Grb10 isoforms. We will utilize this information to address the function of Grb10 based on its known intermolecular interactions and the established roles of its cellular partners.

6.1. Interaction of Grb10 with receptor tyrosine kinases

Grb10 has been reported to interact with a number of receptor tyrosine kinases including the EGF, insulin, IGF-I, ELK, PDGF and Ret receptors (2, 3, 15, 67, 68, 17, 61, 19). Grb10 demonstrated a preference for binding to the insulin receptor when compared to other selected receptor tyrosine kinases, suggesting that Grb10 association with some receptors may not necessarily be functionally relevant (19, 20). A role of Grb10 in insulin signaling is strongly suggested by various data (13, 14) including the observation that Grb10 isoforms (mRNA and protein) are differentially expressed in insulin target cells such as skeletal muscle, adipocytes and human hepatic cancer cell lines (18).

Both the SH2 (2, 17) and BPS (63) domains have been reported to mediate the association of full length Grb10 with the IR. Conflicting reports have been published regarding the SH2 binding site on the IR. Initially, the mouse Grb10 SH2 domain was reported to associate with Y1334 at the carboxyl terminus of the IR (16). In contrast, another group reported that Y1334 is not essential for binding of the SH2 domain from hGrb10gamma (GRB10/IR-SV1) and identified residues Y1162/Y1163 in the kinase activation loop as the SH2 domain binding site on the IR (17). As the SH2 domain of mouse and human shares 99% amino acid sequence similarity, this discrepancy is unlikely to be due to evolutionary divergence. Data from other groups also support Y1162/Y1163 as the Grb10 SH2 binding site on the insulin receptor (53, 63). However, recent crystal structural data predicted that Grb10 binds Y1158 via the dimeric SH2 domain and that the observed dependence on Y1162/Y1163 is probably due in part to the role these phosphotyrosines play in stabilizing the activation loop conformation (59). This would explain why mutation of Y1162/Y1163 disrupted binding by the individual SH2 and BPS domains (63), which associate with residues in the activation loop of the IR kinase domain. While individual reports have been conflicting, a consensus is emerging to suggest that Grb10 binds to phosphotyrosine/s in the activation loop of the IR kinase domain via its SH2 domain, resulting in a conformational change in the IR kinase domain. This in turn creates binding site/s for the BPS domain of Grb10, further strengthening the interaction between Grb10 and the IR. Nonetheless, these findings do not rule out a role of other reported IR binding sites, which remains to be clarified.

Although the activated kinase domains of IR (69) and IGF-IR (70) adopt a very similar overall structure, the Grb10 binding site on IGF-IR appears to differ from IR. Yeast 2-hybrid studies indicated that mGrb10 did not interact with either beta subunit of the IGF-IR mutated at K1003 (ATP-binding site), or a receptor truncated at amino acid 1229 (68). As mGrb10 interacted with the beta subunit of the receptor truncated at amino acid 1245, these results
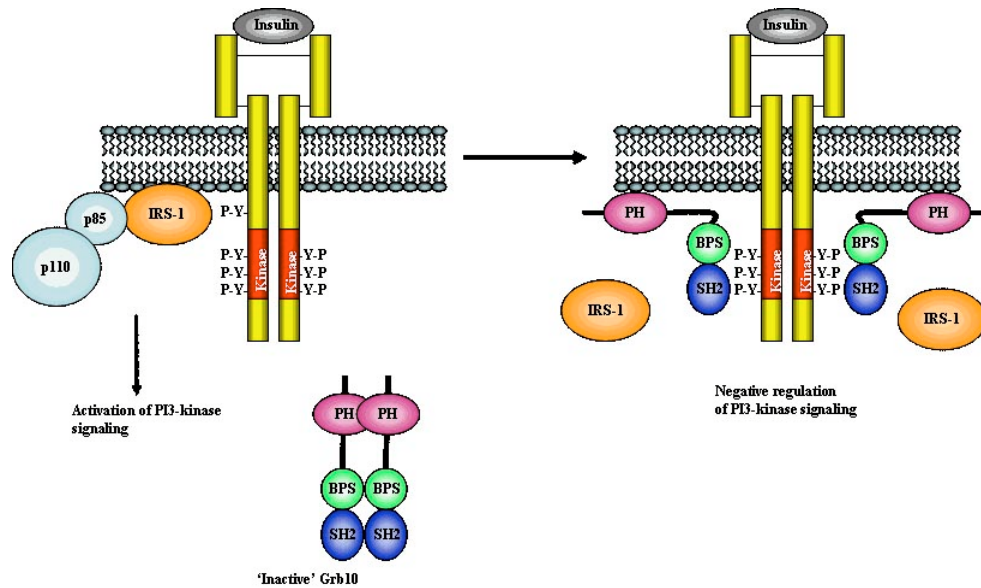
Figure 3. Model of Grb10 inhibition of insulin signaling by blocking the physical access of IRS-1 to the kinase domain of the insulin receptor. Insulin binding to its receptor triggers autophosphorylation of tyrosine residues which then create binding sites for proteins such as IRS-1. IRS-1 binds to the phosphotyrosine residues in the kinase domain of the IR and recruits the regulatory subunit (p85) of PI 3-kinase to the receptor complex. The binding of the p85 subunit to IRS-1 results in activation of the catalytic subunit (p110) of PI 3-kinase and production of phosphorylated lipid second messengers. Grb10 may exist in an 'inactive' state due to oligomerization, activation of Grb10 could occur by phosphorylation, resulting in loss of auto-inhibition. Grb10 then binds to the phosphorylated tyrosines in the IR kinase domain and physically restrict access of IRS-1 to the IR kinase domain.

indicated that mGrb10 binds to autophosphorylated tyrosine residues located between amino acids 1229 and 1245 at the C-terminus of IGF-IR (68). In contrast, an independent study demonstrated that the SH2 domain of mGrb10 did not bind to the mutant IGF-IR[Y1316F], indicating that Y1316 of the IGF-IR is essential for Grb10 binding (12). Both reports are inconsistent with an earlier publication demonstrating that the C-terminus of IGF-IR is not required for interaction with the SH2 domain from human Grb10 (53). It is conceivable that Grb10 interacts with IR and IGF-IR in a comparable mechanism involving the activation loop as well as C-terminal sequences. To this date, only the important association involving the activation loop of IR has been characterized. It remains to be verified whether Grb10 binds to the kinase domain of IGF-IR in a manner similar to the IR.

6.1.1. Effect of Grb10 on insulin signal transduction

The peptide hormones insulin and IGF-I are important for the normal control of metabolic and growth related processes (71-74). Under non-disease conditions, IR signaling primarily regulates metabolic functions including protein synthesis, glucose uptake and glycogen synthesis, whereas the IGF-I receptor signaling mediates growth and differentiation (75). Defects in insulin and IGF-I signaling pathways have been implicated in the development of diabetes and cancer, respectively.

Evidence for a negative role of human Grb10 in insulin signaling surfaced early (2). Overexpression of the PH domain truncated isoform hGrb10beta in Chinese Hamster Ovary cells overexpressing the insulin receptor (CHO/IR) resulted in partial inhibition of tyrosine

phosphorylation of IRS-1 and the GTPase-activated protein p60, and partial inhibition of PI 3-kinase activity. In addition, binding of Grb10 via its SH2/BPS domains has been shown to inhibit the catalytic activity of the IR *in vitro* (13, 66, 76). Therefore, the data suggests that down-regulation of IRS-1/PI 3-kinase signaling is a result of inhibition of the catalytic activity of the IR due to the binding of Grb10 to the kinase domain regulatory loop. However, the possibility that Grb10 may actually prevent substrate access rather than directly inhibiting the IR kinase activity was not addressed. Recently, yeast-tri-hybrid studies demonstrated that full length human Grb10zeta blocks association between the IRS proteins and IR in a SH2 domain-dependent manner (77). While Grb10 inhibited insulin-stimulated IRS-1 tyrosine phosphorylation in a dose-dependent manner, the IR kinase activity towards its autophosphorylation sites was not affected. Taken together, the data suggest that Grb10 inhibition of downstream insulin signaling through the IRS/PI 3-kinase/Akt pathway may be achieved by physically blocking IRS access to the IR (77) (Figure 3). In addition to inhibiting insulin-induced activation of the PI 3-kinase pathway, hGrb10 overexpression also suppresses activation of the MAPK pathway (Langlais et al, submitted).

In contrast, independent findings indicate that Grb10 has a positive effect on insulin signaling (5). Overexpression of mGrb10delta in mouse L6 cells or delivery of cell membrane permeable peptide mimetics representing entire mGrb10delta into differentiated 3T3-L1 adipocytes stimulated metabolic and enzymatic insulin responses, while individual Grb10 peptide mimetics representing the proline-rich region or the SH2 domain

blocked all respective insulin responses (14). Mechanistically, mGrb10 was found to associate with p85 and regulate PI 3-kinase activity whereas no regulation of IR, IRS-1, or IRS-2 phosphorylation was observed (14).

A possible explanation for the observed discrepancy in the effects of Grb10 on insulin action may be that the signal elicited by Grb10 expression, whether stimulatory or inhibitory, may be determined by the exact balance of signaling mediators in the experimental cell system.

6.1.2. Effect of Grb10 on biological events downstream of IR/IGF-IR

Grb10 has been reported to play a positive or negative role in regulating insulin and IGF-I induced mitogenesis, depending on the specific experimental approach and/or cellular context. Microinjection of purified human Grb10 SH2+BPS domains fused to GST into mammalian cells inhibited insulin and IGF-I mediated mitogenesis by approximately 50 % (17, 63). If this inhibition was due to competition with endogenous Grb10, this result may indicate that Grb10 plays a positive role in regulating insulin/IGF-I signaling. Similarly, cell membrane-permeable fusion peptide mimetics of the individual mGrb10 SH2 and BPS domains or proline-rich region inhibited insulin and IGF-I mediated DNA synthesis (12). Although the 16 aa proline-rich Grb10 peptide mimetic significantly interfered with insulin- and IGF-I-mediated mitogenesis, it had no effect on PDGF-mediated mitogenesis as measured in NIH 3T3 or baby hamster kidney (BHK) fibroblasts by DNA synthesis or cell proliferation, suggesting its functional specificity (12). By comparison, a SH2 domain peptide mimetic blocked, and overexpressed mGrb10delta stimulated all three hormone responses. These observations suggest a differential role of the Grb10 proline-rich region in selected mitogenic signals consistent with a dominant-negative role of individual Grb10 proline-rich or SH2 domain peptide mimetics (12). Similarly, the proline-rich or SH2 domain peptide mimetics substantially interfered with key insulin-stimulated metabolic responses including lipogenesis, glycogen synthesis, glucose and amino acid uptake, all of which were stimulated by increased levels of full length mGrb10delta (14). Mechanistically, the peptides interfered with insulin activation of key enzymes involved in the insulin signaling pathway and with the direct association between p85 and mGrb10. The combined data indicate a stimulatory role of mGrb10delta in specific key metabolic and mitogenic signaling pathways, and implicate a role of the SH2 domain in all regulated mechanisms and of the proline-rich regions in selected signaling mechanisms excluding the mitogenic response to PDGF (5).

However, the SH2 domain of Grb10, unlike other SH2 domains, binds to the kinase domain of the insulin receptor, and likewise, the BPS domain also binds to the kinase domain of the IR and IGF-IR (63). Therefore, either domain could conceivably mimic the function of full length Grb10 in inhibiting insulin signaling rather than act as a dominant negative. More compelling evidence for a positive role of Grb10 in insulin/IGF-I signaling has

emerged from studies in which overexpression of the entire mGrb10delta stimulated insulin and IGF-I induced DNA synthesis in a dose-dependent manner, and promoted cell proliferation in mouse NIH 3T3 cells (12). Similarly, mGrb10delta was shown to play a positive role in stimulating insulin-induced metabolic responses such as glycogen synthesis, glucose and amino acid uptake, and lipogenesis in 3T3-L1 adipocytes (14).

In contrast, a role of Grb10 as a negative regulator of IGF-I signaling has been reported (11). Cell lines expressing mGrb10delta (derived from IGF-I receptor gene-deficient mouse cells expressing human IGF-IR) exhibited a dose-dependent inhibition of IGF-I induced cell proliferation. Grb10 has also been reported to play a negative role in regulating glycogen synthase activity and glycogen synthesis in primary rat hepatocytes (13). Overexpression of hGrb10zeta resulted in 50 % inhibition of insulin stimulated IR autophosphorylation and activation. However, in contrast to the effects observed in Chinese Hamster Ovary Cells overexpressing the IR (2) and in differentiated 3T3-L1 adipocytes (77), overexpression of Grb10 in primary rat hepatocytes did not affect insulin-induced IRS-1/2 phosphorylation nor PI 3-kinase activity (13). This discrepancy may possibly be explained by cell line-specific differences (CHO/IR and 3T3-L1 adipocytes versus primary rat hepatocytes). Intriguingly, partial reduction of IR autophosphorylation, glycogen synthase activity, and glycogen synthesis induced by hGrb10zeta over-expression did not correspond with a reduction in insulin-stimulated IRS-1 phosphorylation, PI 3-kinase activation, Akt/PKB activity or GSK3 activity, leading to the interpretation that Grb10 is inhibiting a novel and unidentified insulin signaling pathway in these hepatocytes (13).

6.1.3. Interaction of Grb10 with other receptor tyrosine kinases

A yeast-2-hybrid screen of a mouse embryonic library using the cytoplasmic domain of ELK, an Eph receptor family member, as bait yielded the SH2 domain of mGrb10 (61). Eph family members have been implicated in regulating vascular development, tissue-border formation, cell migration, axon guidance and synaptic plasticity (78). Yeast-2-hybrid studies and *in vitro* binding assays showed that the association of Grb10 with the cytoplasmic tail of ELK was dependent upon the kinase activity and autophosphorylation of ELK at Y929 (61). Ligand stimulation of HRMEC (human renal macrovascular endothelial cells) induced ELK autophosphorylation and binding of endogenous Grb10 isoforms. However, it is not known whether the association of Grb10 with ELK results in Grb10 phosphorylation by ELK and whether this association has an impact on ELK downstream signaling.

A yeast-2-hybrid screen of a mouse embryonic library using the cytoplasmic domain of the murine Ret tyrosine kinase receptor as bait identified the SH2 domain of mGrb10 (15). This interaction was confirmed *in vitro* and in 293 T cells. Ret has been shown to play critical roles in renal development and development of endocrine organs

derived from the neural crest such as the adrenal medulla and the thyroid gland (79, 80). The SH2 domain of Grb7, which shares 64 % sequence identity to the Grb10 SH2 domain, has also been demonstrated to bind to Ret (81). In addition, the SH2 domain of Grb7 possesses a higher affinity for Ret compared to Grb10's SH2 domain and Grb7 undergoes tyrosine phosphorylation in response to Ret activation. The Grb10 binding site on Ret was later identified to be Y429, however, the ability of the Ret^{Y429F} to induce DNA synthesis was unimpaired, suggesting against a role of Grb10 in regulating the mitogenic activity of this receptor (67).

6.2. Interaction of Grb10 with non-receptor tyrosine kinases

In addition to receptor tyrosine kinases, Grb10 interacts with cellular signaling molecules such as Janus kinase 2 (Jak2) (60), the oncogenic tyrosine kinase Bcr-Abl (45), Tec tyrosine kinase (82), MEK and Raf-1 (21), and Akt (83).

6.2.1. Bcr-Abl and oncogenesis

Bcr-Abl is an oncoprotein produced by the fusion of a region from the Bcr Ser/Thr kinase to the tyrosine kinase Abl due to the juxtaposition of the breakpoint cluster region (bcr) on chromosome 22 to the c-Abl gene on chromosome 9 (84). Several isoforms of Bcr-Abl, varying by how much of the Bcr sequence is fused to Abl, are associated with chronic myelogenous leukemia (CML) and acute lymphoblastic leukemia (ALL) (reviewed in (85)). The oncogenic properties of Bcr-Abl are attributed to its ability to activate different pathways including the PI3-kinase, MAP-kinase and STAT signaling pathways.

A novel pathway involved in the transforming ability of Bcr-Abl was identified in a modified yeast-2-hybrid screen with phosphorylated Bcr-Abl as bait (45). Bcr-Abl was found to associate with the SH2 domain of Grb10 from a mouse embryonic cDNA library and a library constructed from a CML cell line. The Grb10 SH2 domain failed to interact with a kinase-defective Bcr-Abl, demonstrating the phosphotyrosine-dependency of this interaction. Complete Bcr-Abl interacted with Grb10 *in vitro*, *in vivo* and in yeast, and this interaction appeared to be mediated by amino acids 242-446 of Bcr and the SH2 domain of Grb10. Overexpression of full length Bcr-Abl in bone-marrow derived Ba/F3 cells imparted interleukin-3 (IL-3) independent proliferation (86). In comparison, expression of a Bcr-Abl deletion mutant lacking the ability to bind Grb10 in these cells resulted in a weaker transforming potential and delayed the onset of IL-3 independent growth, suggesting that this interaction is important but not essential for the induction of Bcr-Abl induced, IL-3 independent growth in these cells (45).

6.2.2. Tec and regulation of cytokine signaling

A role of Grb10 in the regulation of cytokine signaling in lymphocytes was supported by the observation that hGrb10beta associates with the kinase domain of Tec in a yeast-2-hybrid screen for novel Tec-interacting proteins (82). This interaction was verified in 293 cells by co-immunoprecipitation studies of overexpressed proteins.

Although the association between the two proteins is independent of the Tec autophosphorylation state, it resulted in hGrb10beta phosphorylation at Tyr-67 by Tec.

Many members of the Tec tyrosine kinase family play important roles during antigen receptor signaling and lymphocyte development (see (87) for review). Overexpression of Grb10 inhibited IL-3 stimulated and Tec-mediated expression of the luciferase reporter gene from the *c-fos* promoter in Ba /F3 cells in a Grb10 dose-dependent manner (82). Overexpression of the phosphorylation site mutant Grb10^{Y67F} also attenuated IL-3 induced *c-fos* transcription, albeit to a lesser extent. Therefore, it appears that phosphorylation of Grb10 by Tec results in attenuation of Tec signaling. As the kinase activity of Tec is not reduced by co-expression of Grb10, it is possible that Grb10 exerts its effect on downstream effectors of Tec in a negative feedback loop to down-regulate Tec signaling.

6.2.3. The MAPK pathway and apoptosis

An interaction between the SH2 domain of hGrb10zeta with MAPK kinase kinase MEK1 was detected when the latter was used as bait in a yeast-2-hybrid screen of a human fetal cDNA library (21). *In vitro* binding studies further confirmed the interaction of the Grb10 SH2 domain and full length hGrb10zeta with MEK1. Both forms also associated with Raf1 *in vitro*, but not with MEK4 (SEK1/ JNKK/MKK4), a related protein which shares 63 % amino acid sequence similarity with MEK1. The *in vitro* association of hGrb10zeta with MEK1 and Raf1 was independent of tyrosine phosphorylation. Treatment with alkaline phosphatase inhibited this interaction, indicating that either the Grb10 SH2 domain recognizes a phosphothreonine- or phosphoserine-containing sequence, or that Grb10 has lower affinities for the altered conformation of the non-phosphorylated kinases.

Deletion mapping using the yeast-2-hybrid system revealed that the Grb10 SH2 domain binds to the amino-terminus of Raf1 (amino acids 1-330) and carboxyl-terminus of MEK1 (last 88 amino acids) (21). MEK1 bound to Grb10 in several different cell lines, in comparison to the Raf1-Grb10 interaction which appeared to be cell-line specific. In addition, the Raf1-Grb10 association appeared to be constitutive, whereas the binding of MEK1 to Grb10 was growth factor dependent. Overexpression of Grb10 containing point mutations in the SH2 domain, which abolishes interaction with either MEK1 or tyrosine kinase receptors, induced apoptosis in two different cell lines. The apoptotic phenotype was reversed by overexpression of wild-type Grb10, suggesting that these mutants act by sequestering necessary signaling components.

Generation of a rabbit polyclonal antibody against purified recombinant hGrb10zeta protein (R520L) (88) helped addressed the functional significance of the Grb10-Mek1 association. This antibody specifically recognized Grb10 and not Grb7 or Grb14. Surprisingly, immunocytochemistry studies revealed that endogenous

Grb10 is localized to the mitochondria in COS-1 and HeLa cells, in contrast to overexpressed Grb10 which is cytoplasmic. It appears that overexpression of Grb10 causes an alternate localization to the cytoplasm. Serum or IGF-I stimulation induced transient membrane translocation of a small fraction of endogenous Grb10, consistent with the ability of this protein to bind receptor tyrosine kinases upon growth factor stimulation. The mitochondrial localization of endogenous Grb10 is supported by cell fractionation studies, which also revealed the presence of Raf1 and MEK1 in mitochondrial extracts (88). Accordingly, endogenous mitochondrial Raf-1 co-immunoprecipitated endogenous Grb10 from mitochondrial protein extracts. Furthermore, Grb10's affinity for Raf-1 is increased in mitochondrial extracts purified from UV-treated cells. As Raf-1 has been reported to possess anti-apoptotic properties, it was proposed that Grb10 regulates programmed cell death by modulating the activity of mitochondrial Raf-1. Thus, Grb10 may serve as a link between cell surface receptors and the apoptotic machinery on the outer mitochondrial membrane, probably in collaboration with the PI 3-kinase/Akt signaling pathway.

6.2.4. Modulation of c-kit signaling

Stem cell factor (SCF) is a growth factor critical for hematopoiesis and the generation of melanocytes and germ cells (see review in (89)).The binding of SCF to its receptor tyrosine kinase, c-kit, activates multiple signal transduction pathways including the PI 3-kinase, JAK/STAT and Ras-Raf-MAP kinase pathways. Part of the BPS and the entire SH2 domain of mouse Grb10 were found to bind to the cytoplasmic tail of c-kit in a yeast-2-hybrid screen (83). The interaction of the mGrb10 SH2 domain with c-kit was verified *in vitro*, and endogenous mGrb10 was found to associate with c-kit in Moe7 cells in a SCF-dependent manner.

In this study, mGrb10 was also shown to be constitutively associated with Akt, indicating that mGrb10 could play a role in mediating the translocation of Akt to the plasma membrane. It was also demonstrated that overexpression of mGrb10 resulted in the activation of Akt in a PI 3-kinase-independent, and SH2-domain dependent fashion. Furthermore, co-expression studies demonstrated that that mGrb10 and c-kit have a synergistic effect on Akt activation.

6.3. Interaction of Grb10 with other signaling molecules
6.3.1. Regulation of Growth Hormone (GH) signaling

By employing a modified version of the cloning of receptor targets (CORT) strategy, the SH2 domain of Grb10 was found to associate with the Elk-phosphorylated cytoplasmic tail of the Growth Hormone receptor (GHR) (60). This receptor regulates the expression of a broad range of genes responsible for growth, metabolism and cellular differentiation. Ligand binding induces receptor homo-dimerization and binding of Janus kinase 2 (Jak2), resulting in Jak2 autophosphorylation and Jak-2 mediated phosphorylation of the GHR cytoplasmic domain. Proteins containing SH2 domains such as the STAT family, Shc and IRS-1/2 then bind to the phosphorylated tyrosine residues of the GHR and are phosphorylated by Jak2, leading to the activation of signal transduction pathways regulating transcription, growth and metabolic activity, respectively (90).

The association between endogenous hGrb10 and endogenous GHR was verified by co-immunoprecipitation studies in human hepatoma (Huh-7) cells. This association was also observed in a reciprocal experiment in 293 cells where overexpressed GHR was able to co-immunoprecipitate mGrb10 in the presence of Jak2 (60). A truncated form of GHR lacking the cytoplasmic tail was still able to interact with overexpressed mGrb10 in the presence of exogenous Jak2, suggesting that mGrb10 may also be interacting with Jak2. Direct interaction of mGrb10 with Jak2 was confirmed by GST-pulldown studies. Although overexpression of mGrb10 had no effect on GHR, Jak2 or STAT5 phosphorylation, functional tests revealed Grb10 overexpression inhibited transcription of reporter genes containing the serum response element of *c-fos* and the GH response element 2 of the *Spi2.1* gene. These results indicate a novel role for Grb10 in negatively regulating some GH signaling pathways downstream of Jak2 and independently of STAT5.

6.3.2. Nedd4 and regulation of IGF-IR stability

Mouse Nedd4 (mNedd4-1, neuronal precursor cell-expressed developmentally down-regulated 4) was identified as a mGrb10 associated protein in a yeast-2-hybrid screen of a mouse embryonic cDNA library (62). Nedd4 belongs to a family of proteins which shares a Ca^{2+}/lipid-binding domain (C2) at the N-terminus, several WW domains which bind to proline-rich sequences, and a HECT (homologous to the E6-AP carboxyl-terminus) domain (91). The presence of the HECT, also known as a ubiquitin-ligase domain, results in classification of Nedd4 family members as ubiquitin-ligases (E3). Ubiquitin-ligases (E3) represent the substrate-specific and final step of the ubiquitin pathway, in which they catalyze the transfer of ubiquitin from the conjugating enzyme (E2) to the substrate protein (reviewed in (91, 92)). Ubiquitination typically labels proteins for rapid degradation (reviewed in (93)).

The interaction of mNedd4-1 with mGrb10 is mediated by the C2 domain of Nedd4, rather than the WW protein interaction domain (62). Both the individual SH2 and BPS domains of mGrb10 can associate with mNedd4-1, although the SH2 domain exhibited higher affinity. mNedd4-1 is constitutively associated with mGrb10, and endogenous Nedd4 forms a complex with Grb10 in a Ca^{2+} and phosphotyrosine-independent manner, although this does not result in Grb10 ubiquitination (62). Instead, the Grb10/Nedd4 complex regulates ubiquitination and stability of the IGF-I receptor (24). A model for the role of Grb10 as an adapter protein in connecting Nedd4 to the IGF-IR has emerged (24). Association of Grb10 to Nedd4 is constitutive and occurs via binding of the SH2 domain of mGrb10 to the C2 domain of Nedd4. IGF-I treatment results in binding of the BPS domain of Grb10 to the activated IGF-IR, bringing Nedd4 in close proximity to the receptor and resulting in ubiquitination and subsequent degradation of IGF-IR.

This is the first report indicating a role for the Nedd4 family of E3 ubiquitin ligases in the regulation of ubiquitination, internalization and stability of tyrosine receptor kinases. It remains to be established whether

Grb10 plays a similar role in the ubiquitination and stability of other associated tyrosine receptor kinases such as the IR.

6.3.3. Regulation of potassium channel activity

A role of Grb10 has been implicated in the regulation of the Kv1.3 voltage-gated potassium channel (94). Kv1.3 belongs to the *Shaker* family of membrane spanning channel proteins with K$^+$-selective pores and is highly expressed in T-lymphocytes, the dentate gyrus of the hippocampus, the pyriform cortex, and the olfactory bulb (OB) (95, 94). The biophysical properties of Kv channels are regulated by conformational changes induced by phosphorylation (96) and possibly by protein-protein interactions with intracellular partners (97-100). The overexpressed *Shaker* family K$^+$ channel proteins Kv1.3 and Kv1.5 are phosphorylated by the v-Src kinase, resulting in modulation of current magnitude and kinetic properties, but unlike Kv1.5, a direct interaction between Kv1.3 and v-Src was not detected (98).

Grb10 overexpression significantly reduces v-Src-induced Kv1.3 tyrosine phosphorylation, current suppression, and disrupted cumulative inactivation properties, suggesting that Grb10 plays a role in the modulation of v-Src induced Kv1.3 channel function (94). This infers that Grb10 plays a role in interfering with v-Src phosphorylation of Kv1.3. On the other hand, Grb10 appears to modulate Kv1.3 activity in a phosphorylation-independent manner since co-expression of Grb10 with a kinase-impaired v-Src mutant (R385A) resulted in altered current magnitude and inactivation kinetics. It should be noted that some effects, such as the shift in voltage dependence of v-Src on Kv1.3 function, were not altered by Grb10 overexpression. Thus Grb10 modulation of the v-Src kinase-activated Kv1.3 potassium channel appears to be complex and may involve multiple mechanisms and sites of interaction (94). Further studies are needed to address whether Grb10 alone (without v-Src overexpression) will have an effect on the basal properties of the Kv-channel activity, and whether direct protein-interactions can be demonstrated between the Kv-channel and Grb10.

The following model has been suggested (94). Kv1.3 and Grb10 proline-rich regions may compete for interaction with the Src SH3 domain such that this domain preferentially binds to a Grb10 proline-rich target, allowing Src access to the Kv1.3 ion channel only in the absence of Grb10 expression or its alternate subcellular localization. Grb10 regulates v-Src modulation of Kv1.3 by blocking the access of the kinase to the ion channel and inhibiting phosphorylation. This permits normal Kv1.3 current magnitude and cumulative inactivation in the presence of v-Src but voltage-dependence remains modulated by v-Src.

7. REGULATION OF GRB10 FUNCTION

7.1. Phosphorylation

When compared to Grb7 or Grb14, a greater number of protein kinases has been implicated as binding partners of Grb10, suggesting that this adaptor protein may be regulated by phosphorylation. Although Grb10 has been reported to bind to receptor tyrosine kinases such as the IR, IGF-IR, EGF, and VEGF, **direct** phosphorylation of Grb10 by these receptors has not been reported. In the case of IR mediated phosphorylation, it was demonstrated that Grb10 was not directly phosphorylated by the IR either in vitro (101) or in cells (82). Growth factor stimulation has been reported to induce either serine phosphorylation or tyrosine phosphorylation of Grb10. Serine phosphorylation of hGrb10zeta and mGrb10 in response to insulin (18) and EGF (3) stimulation, respectively, has been reported. The beta, gamma and zeta isoforms of hGrb10 have been demonstrated to be phosphorylated on tyrosine residues upon insulin treatment (19, 101). The identity of the serine kinase responsible for the insulin-induced serine phosphorylation of Grb10 remains to be determined. MAP kinase is a potential candidate because 1) treatment with a MAPK inhibitor suppressed the basal phosphorylation of hGrb10zeta (2, 18) MAPK can phosphorylate hGrb10zeta on serine residues in vitro (Langlais et al, unpublished data).

On the other hand, the Tec, Src, and Fyn tyrosine kinases have been shown to phosphorylate Grb10 isoforms. Tec was found to associate with Grb10 in a yeast-2-hybrid screen (see section 6.2.2 and (82)) and phosphorylates Grb10 at Tyr 67 in 293 cells. The Grb10^{Y67F} mutant partially lost the ability to suppress IL-3 induced transcription from the c-fos promoter, indicating that phosphorylation of Grb10 at Tyr 67 is important for this suppression. The mechanism of Grb10 suppression of IL-3 induced c-fos transcription remains unclear. As the kinase activity of Tec is not affected by co-expression of Grb10, it is unlikely that Grb10 negatively regulates the c-fos activation pathway through a feedback loop involving Tec (82). A possibility that remains unexplored is that Grb10 may sequester other positive signaling components in the c-fos activation pathway in a phosphorylation-dependent manner.

Pre-treatment of CHO/IR cells expressing hGrb10zeta with herbimycin B, an inhibitor of Src/Fyn kinases, blocked insulin- and vanadate-induced tyrosine phosphorylation of Grb10, implicating the involvement of these kinases in Grb10 phosphorylation (101). Constitutively active Src or Fyn stimulated Grb10 phosphorylation whereas overexpression of dominant negative forms of these proteins inhibited insulin-stimulated tyrosine phosphorylation of Grb10. The major Src/Fyn phosphorylation site was identified as Tyr 67, and mutation of Tyr 67 to glycine significantly reduced the insulin-stimulated or Src/Fyn-mediated tyrosine phosphorylation *in vivo*. hGrb10^{Y67G} had a higher affinity for the insulin receptor compared to the wild type protein, indicating that tyrosine phosphorylation of Grb10 negatively regulates its binding to the IR (101). It remains unclear whether insulin stimulates phosphorylation of Grb10 in insulin-responsive target cells such as skeletal muscle and adipocytes when compared to an IR overexpression system, and whether increased association of Grb10^{Y67G} with the insulin receptor results in attenuation of signaling downstream of the IR.

7.2. Dimerization/oligomerization

Gel filtration studies revealed that hGrb10zeta can form tetramers (54). A more recent study indicated that

hGrb10gamma is dimeric in solution, although the possibility that hGrb10gamma forms a compact tetramer rather than an elongated dimer was not excluded (59). A possible explanation for the observed differences may lie in the 58 amino acid extension at the hGrb10zeta N-terminus when compared to hGrb10gamma, which could affect its self-association properties. As the oligomeric properties of hGrb10 appear to be mainly defined by the SH2 and BPS domains, the differences in the N-termini is unlikely to account for the discrepancy (54, 59).

A possible function of Grb10 dimerization/oligomerization may be to maintain the protein in an 'inactive' state that prevents it from binding non-specifically to cellular phosphotyrosine-bearing proteins (54). This hypothesis is supported by the observation that the N-terminus of Grb10, which associates with the BPS/SH2 domain, competitively inhibits binding of hGrb10 to the IR (54). Therefore, the BPS and SH2 domains of oligomeric Grb10 may be unable to interact with other phosphotyrosine-bearing proteins due to self-association with the N-terminus of Grb10. Growth factor stimulation may induce a conformational change in Grb10, thereby relieving the inhibition of the BPS and SH2 domain by the N-terminus, freeing these domains to interact with their respective targets.

The presence of multiple protein-protein interaction domains indicates that Grb10 may function as a scaffold protein to recruit multiple signaling molecules. Consistent with this hypothesis, Grb10 can simultaneously bind to the IR and other cellular signaling proteins (17, 21). If Grb10 functions as a scaffold protein, overexpression of Grb10 may result in sequestration of signaling components, resulting in misleading inhibitory effects. Such a mechanism could help explain discrepancies observed in different cellular systems where Grb10 has been reported to play positive and negative roles. This question deserves major attention in the future.

7.3. Subcellular localization

Overexpression studies revealed that Grb10 is localized to the cytoplasm and translocates to the plasma membrane upon insulin stimulation (18, 19). In contrast, immunocytochemistry of COS-1 and HeLa cells using a rabbit polyclonal antibody raised against complete Grb10zeta (R520L) revealed that endogenous Grb10 is associated with mitochondria (88). Subcellular fractionation studies demonstrated that Grb10 co-localized with mitochondrial markers such as CoxI and Bcl-2 in the mitochondrial-enriched fraction. However, as Grb10 lacks an apparent mitochondrial targeting sequence, the mechanism behind its mitochondrial localization remains uncharacterized. The mitochondrial localization of Grb10 has been suggested to serve as a regulatory mechanism to sequester Grb10 away from its interactive partners and/or play a role in apoptosis (21).

8. CONCLUSIONS AND FUTURE PERSPECTIVES

Despite the increasing amount of information on Grb10 signaling, a clear definition of Grb10 function

as a positive or negative regulator of cellular signaling remains elusive. The lack of clarity of many reports in defining the specific Grb10 isoform under consideration adds to the confusion since the distinct isoforms may play specific roles. Isoform-specific effects may contribute to the discordant data, however, opposite results have been obtained using the same isoform. For example, overexpression of mGrb10 has been shown to inhibit IGF-I-mediated growth, delay the S and G2 phases, and to partially reverse the transformed phenotype of Balb/c 3T3 cells stably expressing human IGF-IR (11). In contrast, mGrb10 overexpression was reported to stimulate insulin, IGF-I and PDGF-1 mediated DNA synthesis in a dose dependent manner and cell proliferation in mouse NIH 3T3 fibroblasts or BHK fibroblasts (12). Possible explanations for this discrepancy may lie in the different cell lines used, a highly transformed overexpression system in the former compared to normal fibroblasts in the latter study, or differences in the experimental endpoints tested. Discrepancies may be exacerbated if Grb10 indeed functions as a scaffold protein and sequesters signaling mediators in distinct cellular systems differentially depending on the availability/ability of these proteins to associate with Grb10, thus promoting or inhibiting cellular signaling.

Overexpression of Grb10 isoforms has frequently been reported by various teams to interfere with or promote growth factor signaling. In addition, Grb10 appears to be involved in the regulation of cytokine signaling, receptor stability, and cell survival (summarized in Figure 4). However, until recently, it has not been established whether endogenous Grb10 plays similar roles. A study in which the maternally imprinted mouse Grb10 gene is disrupted has made a significant contribution towards clarifying Grb10 function. Loss of maternally inherited Grb10 function leads to fetal and placental overgrowth, resulting in larger Grb10 knockout neonates with disproportionate overgrowth of the liver and relative sparing of the brain (22). This animal model establishes the role of Grb10 as a potent growth inhibitor. The enlarged liver was caused by increased glycogen accumulation, and taken together with a reported inhibitory role of Grb10 in primary rat hepatocytes in glycogen synthesis (13), the collective data argues for a negative role of Grb10 in regulating mitogenic and possibly metabolic responses.

While data from the Grb10 knockout mice suggested that Grb10 has a negative effect on growth and potentially metabolism (22), signal transduction in cells devoid of Grb10 remains uncharacterized. A future approach is to characterize growth factor responses in tissues from these knockout mice. Another approach is to deplete endogenous Grb10 proteins in cell lines by either the anti-sense or RNA interference approach (reviewed in (102)). Anti-sense RNA technology has been successfully used to lower endogenous Grb7 protein levels in esophageal carcinoma cells, resulting in suppression of the
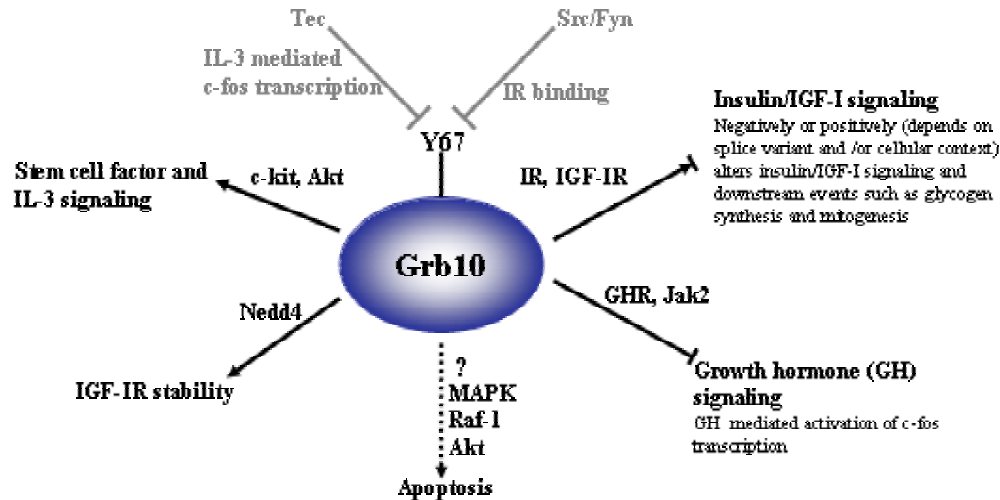
Figure 4. Simplified diagram of Grb10 function in cells. Lines that end with an arrowhead represent a positive effect whereas lines that are capped by another perpendicular line represent inhibition. Tyr 67 (Y67) represents the phosphorylation site of Src/Fyn and Tec kinases (represented by gray lines). Dotted lines represent pathways that are not well characterized.

invasive phenotype of these cells (103). The RNA interference approach was successfully employed to generate a HeLa/IR cell line in which endogenous Grb10 expression is suppressed (Langlais and Liu, unpublished data). Preliminary data from this study shows that depletion of hGrb10 results in increased activation of insulin-stimulated MAPK and PI 3-kinase signaling pathways, which correlates with the knockout mice model and supports a negative role for Grb10 in growth factor signaling.

Although Grb10 proteins interact with a plethora of receptor and non-receptor protein kinases, only Tec, Src and Fyn have been reported to phosphorylate Grb10 on Tyr67. In addition, the identities of the Ser/Thr kinase/s that phosphorylate Grb10 remain elusive. More work is required to determine whether the associated kinases phosphorylate Grb10, to identify the specific site/s of phosphorylation, and to determine whether phosphorylation affects Grb10 function.

A proven effective strategy to study the function of Grb10 is based on the identification of its interacting partners. For example, mGrb10 was found to interact with the ubiquitin ligase, Nedd4, in a yeast-2-hybrid screen using full length mGrb10 as bait (62). This interaction was later demonstrated to regulate the ligand-induced ubiquitination and stability of IGF-IR (24). Therefore, identification of additional binding partners using the yeast-2-hybrid, *in vitro* binding, and co-immunoprecipitation techniques, in conjunction with mass spectrometry studies should help elucidate the function of Grb10.

Another future approach to study Grb10 function lies in the utilization of transgenic mice. Current data indicate that Grb10 plays an important role in insulin and IGF-I signaling, and alterations in Grb10 function may

contribute to the development of diseases such as type II diabetes and cancer. Increased dosage of Grb10 has been linked to the growth suppressive effects observed in Silver-Russell individuals. In this context, elucidation of the molecular mechanisms of Grb10 signaling may ultimately lead to the development of new therapeutic strategies.

9. ACKNOWLEDGEMENTS

10. REFERENCES

1. Margolis, B., O. Silvennoinen, F. Comoglio, C. Roonprapunt, E. Skolnik, A. Ullrich, and J. Schlessinger: High-efficiency expression/cloning of epidermal growth factor-receptor-binding proteins with Src homology 2 domains. *Proc. Natl. Acad. Sci. USA* 89, 8894-8898. (1992)
2. Liu, F. and R.A. Roth: Grb-IR: A SH2-domain-containing protein that binds to the insulin receptor and inhibits its function. *Proc. Natl. Acad. Sci., USA.* 92, 10287-10291. (1995)
3. Ooi, J., V. Yajnik, D. Immanuel, M. Gordon, J.J. Moskow, A.M. Buchberg, and B. Margolis: The cloning of Grb10 reveals a new family of SH2 domain proteins. *Oncogene* 10, 1610-1630. (1995)
4. Daly, R.J., G.M. Sanderson, P.W. Janes, and R.L. Sutherland: Cloning and characterization of Grb14, a novel

member of the Grb7 gene family. *J. Biol. Chem.* 271, 12502-12510 (1996)

5. Reidel, H. and Braun, *Grb10 in peptide hormone action*, in *Insulin Signaling: From Cultured Cells to Animal Models*, G. Grunberger and Y. Zick, Editors: Taylor and Francis, London 89-105 (2001)

6. Manser, J., C. Roonprapunt, and B. Margolis: C. elegans cell migration gene mig-10 shares similarities with a family of SH2 domain proteins and acts cell nonautonomously in excretory canal development. *Dev Biol* 184(1), 150-164 (1997)

7. Manser, J. and W.B. Wood: Mutations affecting embryonic cell migrations in Caenorhabditis elegans. *Dev Genet* 11(1), 49-64 (1990)

8. Han, D.C., T.L. Shen, and J.L. Guan: The Grb7 family proteins: structure, interactions with other signaling molecules and potential cellular functions. *Oncogene* 20(44), 6315-6321 (2001)

9. Wojcik, J., J.A. Girault, G. Labesse, J. Chomilier, J.P. Mornon, and I. Callebaut: Sequence analysis identifies a ras-associating (RA)-like domain in the N-termini of band 4.1/JEF domains and in the Grb7/10/14 adapter family. *Biochem Biophys Res Commun* 259(1), 113-120 (1999)

10. Ponting, C.P. and D.R. Benjamin: A novel family of Ras-binding domains. *Trends Biochem Sci* 21(11), 422-425 (1996)

11. Morrione, A., B. Valentinis, M. Resnicoff, S.-Q. Xu, and R. Baserga: The Role of mGrb10α in Insulin-like Growth Factor I-mediated Growth. *J. Biol. Chem.* 272, 26382-26387 (1997)

12. Wang, J., H. Dai, N. Yousaf, M. Moussaif, Y. Deng, A. Boufelliga, O.R. Swamy, M.E. Leone, and H. Riedel: Grb10, a positive, stimulatory signaling adapter in platelet-derived growth factor BB-, insulin-like growth factor I-, and insulin-mediated mitogenesis. *Mol. Cell. Biol.* 19, 6217-6228. (1999)

13. Mounier, C., L. Lavoie, V. Dumas, K. Mohammad-Ali, J. Wu, A. Nantel, J.J. Bergeron, D.Y. Thomas, and B.I. Posner: Specific inhibition by hGRB10zeta of insulin-induced glycogen synthase activation: evidence for a novel signaling pathway. *Mol Cell Endocrinol* 173, 15-27. (2001)

14. Deng, Y., S. Bhattacharya, O.R. Swamy, R. Tandon, Y. Wang, R. Janda, and H. Riedel: Grb10 as a partner of PI 3-kinase in metabolic insulin action. *J Biol Chem*, (2003)

15. Pandey, A., H. Duan, P.P. Di Fiore, and V.M. Dixit: The Ret receptor protein tyrosine kinase associates with the SH2-containing adaptor protein Grb10. *J. Biol. Chem.* 270(37), 21461-21463. (1995)

16. Hansen, H., U. Svensson, j. Zhu, L. Laviola, F. Giorgino, G. Wolf, R.J. Smith, and H. Riedel: Interaction between the Grb10 SH2 domain and the insulin receptor carboxyl terminus. *J. Biol. Chem.* 271, 8882-8886. (1996)

17. O'Neill, T.J., T.W. Rose, T.S. Pillay, K. Hotta, J.M. Olefsky, and T.A. Gustafson: Interaction of a GRB-IR splice variant (a human GRB10 homolog) with the insulin and insulin-like growth factor I receptors. Evidence for a role in mitogenic signaling. *J. Biol. Chem.* 271, 22506-22513. (1996)

18. Dong, L.Q., H.-Y. Du, S. Porter, J. Kolakowski, L.F., A.V. Lee, L.J. Mandarino, J.B. Fan, D. Yee, and F. Liu: Cloning, Chromosome Localization, Expression, and Characterization of an SH2 and PH Domain-containing Insulin Receptor Binding Protein hGrb10g. *J. Biol. Chem.* 272, 29104-29112 (1997)

19. Frantz, J.D., S. Giorgetti-Peraldi, E.A. Ottinger, and S.E. Shoelson: Human GRB-IRβ/GRB10 Splice variants of an insulin and growth factor receptor-binding protein with PH and SH2 domains. *J. Biol. Chem.* 272, 2659-2667. (1997)

20. Laviola, L., F. Giorgino, J.C. Chow, J.A. Baquero, H. Hansen, J. Ooi, J. Zhu, H. Riedel, and R.J. Smith: The adapter protein Grb10 associates preferentially with the insulin receptor as compared with the IGF-I receptor in mouse fibroblasts. *J. Clin. Invest.* 99, 830-837. (1997)

21. Nantel, A., K. Mohammad-Ali, J. Sherk, B.I. Posner, and D.Y. Thomas: Interaction of the Grb10 Adapter Protein with the Raf1 and MEK1 Kinases. *J. Biol. Chem.* 273, 10475-10484. (1998)

22. Charalambous, M., F.M. Smith, W.R. Bennett, T.E. Crew, F. Mackenzie, and A. Ward: Disruption of the imprinted Grb10 gene leads to disproportionate overgrowth by an Igf2-independent mechanism. *Proc Natl Acad Sci U S A*, (2003)

23. Hikichi, T., T. Kohda, T. Kaneko-Ishino, and F. Ishino: Imprinting regulation of the murine Meg1/Grb10 and human GRB10 genes; roles of brain-specific promoters and mouse-specific CTCF-binding sites. *Nucleic Acids Res* 31(5), 1398-1406 (2003)

24. Vecchione, A., A. Marchese, P. Henry, D. Rotin, and A. Morrione: The Grb10/Nedd4 Complex Regulates Ligand-Induced Ubiquitination and Stability of the Insulin-Like Growth Factor I Receptor. *Mol Cell Biol* 23(9), 3363-3372 (2003)

25. Jerome, C.A., S.W. Scherer, L.C. Tsui, R.D. Gietz, and B. Triggs-Raine: Assignment of growth factor receptor-bound protein 10 (GRB10) to human chromosome 7p11.2-p12. *Genomics* 40, 215-216. (1997)

26. Blagitko, N., S. Mergenthaler, U. Schulz, H.A. Wollmann, W. Craigen, T. Eggermann, H.H. Ropers, and V.M. Kalscheuer: Human GRB10 is imprinted and expressed from the paternal and maternal allele in a highly tissue- and isoform-specific fashion. *Hum Mol Genet* 9(11), 1587-1595 (2000)

27. Miyoshi, N., Y. Kuroiwa, T. Kohda, H. Shitara, H. Yonekawa, T. Kawabe, H. Hasegawa, S.C. Barton, M.Z. Surani, T. Kaneko-Ishino, and F. Ishino: Identification of the Meg1/Grb10 imprinted gene on mouse proximal chromosome 11, a candidate for the Silver-Russell syndrome gene. *Proc. Natl. Acad. Sci., USA* 95, 1102-1107 (1998)

28. McGrath, J. and D. Solter: Completion of mouse embryogenesis requires both the maternal and paternal genomes. *Cell* 37(1), 179-183 (1984)

29. Surani, M.A., S.C. Barton, and M.L. Norris: Development of reconstituted mouse eggs suggests imprinting of the genome during gametogenesis. *Nature* 308(5959), 548-550 (1984)

30. Cattanach, B.M. and M. Kirk: Differential activity of maternally and paternally derived chromosome regions in mice. *Nature* 315(6019), 496-498 (1985)

31. Surani, M.A.: Imprinting and the initiation of gene silencing in the germ line. *Cell* 93(3), 309-312 (1998)

32. Polychronakos, C. and A. Kukuvitis: Parental genomic imprinting in endocrinopathies. *Eur J Endocrinol* 147(5), 561-569 (2002)

33. Cattanach, B.M., C.V. Beechey, C. Rasberry, J. Jones, and D. Papworth: Time of initiation and site of action of the

mouse chromosome 11 imprinting effects. *Genet. Res.* 68, 35-44. (1996)

34. Silver, H.K., Kisayu, W., George, J., Deamer, W.C: Syndrome of congenital hemihypertrophy, shortness of stature, and elevated urinary gonadotrophins. *Pediatrics* 12, 368-376 (1953)

35. Russell, A.: A syndrome of intrauterine dwarfism recognizable at birth with craniofacial dysostosis, disproportionate short arms and other anomalies. *Proc R Soc Med* 47, 1040-1044 (1954)

36. Kotzot, D., S. Schmitt, F. Bernasconi, W.P. Robinson, I.W. Lurie, H. Ilyina, K. Mehes, B.C. Hamel, B.J. Otten, M. Hergersberg, E. Werder, E. Schoenle, and A. Schinzek: Uniparental disomy 7 in Silver-Russell syndrome and primordial growth retardation. *Hum. Mol. Genet.* 4, 583-587. (1995)

37. Eggermann, T., H.A. Wollmann, R. Kuner, K. Eggermann, H. Enders, P. Kaiser, and M.B. Ranke: Molecular studies in 37 Silver-Russell syndrome patients: frequency and etiology of uniparental disomy. *Hum Genet* 100(3-4), 415-419 (1997)

38. Bernard, L.E., M.S. Penaherrera, M.I. Van Allen, M.S. Wang, S.L. Yong, F. Gareis, S. Langlois, and W.P. Robinson: Clinical and molecular findings in two patients with russell-silver syndrome and UPD7: comparison with non-UPD7 cases. *Am J Med Genet* 87(3), 230-236 (1999)

39. Joyce, C.A., A. Sharp, J.M. Walker, H. Bullman, and I.K. Temple: Duplication of 7p12.1-p13, including GRB10 and IGFBP1, in a mother and daughter with features of Silver-Russell syndrome. *Hum Genet* 105(3), 273-280 (1999)

40. Monk, D., E.L. Wakeling, V. Proud, M. Hitchins, S.N. Abu-Amero, P. Stanier, M.A. Preece, and G.E. Moore: Duplication of 7p11.2-p13, including GRB10, in Silver-Russell syndrome. *Am J Hum Genet* 66(1), 36-46 (2000)

41. Eggermann, K., H.A. Wollmann, G. Binder, P. Kaiser, M.B. Ranke, and T. Eggermann: Biparental expression of IGFBP1 and IGFBP3 renders their involvement in the etiology of Silver-Russell syndrome unlikely. *Ann Genet* 42(2), 117-121 (1999)

42. Wakeling, E.L., M.P. Hitchins, S.N. Abu-Amero, P. Stanier, G.E. Moore, and M.A. Preece: Biallelic expression of IGFBP1 and IGFBP3, two candidate genes for the Silver-Russell syndrome. *J Med Genet* 37(1), 65-67 (2000)

43. Hitchins, M.P., D. Monk, G.M. Bell, Z. Ali, M.A. Preece, P. Stanier, and G.E. Moore: Maternal repression of the human GRB10 gene in the developing central nervous system; evaluation of the role for GRB10 in Silver-Russell syndrome. *Eur J Hum Genet* 9(2), 82-90. (2001)

44. Kay, B.K., M.P. Williamson, and M. Sudol: The importance of being proline: the interaction of proline-rich motifs in signaling proteins with their cognate domains. *Faseb J* 14(2), 231-241 (2000)

45. Bai, R.Y., T. Jahn, S. Schrem, G. Munzert, K.M. Weidner, J.Y.J. Wang, and J. Duyster: The SH2-containing adaptor protein Grb10 interacts with BCR-ABL. *Oncogene* 17, 941-948. (1998)

46. Giovannone, B., E. Lee, L. Laviola, F. Giorgino, K.A. Cleveland, and R.J. Smith: Two novel proteins that are linked to IGF-I receptors by the Grb10 adapter and modulate IGF-I signaling. *J Biol Chem*, (2003)

47. Haslam, R.J., H.B. Koide, and B.A. Hemmings: Pleckstrin domain homology. *Nature* 363, 309-310. (1993)

48. Mayer, B.J., R. Ren, K.L. Clark, and D. Baltimore: A putative modular domain present in diverse signaling proteins. *Cell* 73, 629-633. (1993)

49. Lemmon, M.A. and K.M. Ferguson: Pleckstrin homology domains. *Curr Top Microbiol Immunol* 228, 39-74 (1998)

50. Rebecchi, M.J. and S. Scarlata: Pleckstrin homology domains: a common fold with diverse functions. *Annu Rev Biophys Biomol Struct* 27, 503-528 (1998)

51. Maffucci, T. and M. Falasca: Specificity in pleckstrin homology (PH) domain membrane targeting: a role for a phosphoinositide-protein co-operative mechanism. *FEBS Lett* 506(3), 173-179 (2001)

52. Shen, T.L., D.C. Han, and J.L. Guan: Association of Grb7 with phosphoinositides and its role in the regulation of cell migration. *J Biol Chem* 277(32), 29069-29077 (2002)

53. Dong, L.Q., S. Farris, J. Christal, and F. Liu: Site-directed mutagenesis and yeast two-hybrid studies of the insulin and insulin-like growth factor-1 receptors: The src-homology-2 domain-containing protein hGrb10 binds to the autophosphorylated tyrosine residues in the kinase domain of the insulin receptor. *Mol. Endocrinol.* 11, 1757-1765. (1997)

54. Dong, L.Q., S. Porter, D. Hu, and F. Liu: Inhibition of hGrb10 binding to the insulin receptor by functional domain-mediated oligomerization. *J. Biol. Chem.* 273, 17720-17725. (1998)

55. Sadowski, I., J.C. Stone, and T. Pawson: A noncatalytic domain conserved among cytoplasmic protein-tyrosine kinases modifies the kinase function and transforming activity of Fujinami sarcoma virus P130gag-fps. *Mol Cell Biol* 6(12), 4396-4408 (1986)

56. Pawson, T., G.D. Gish, and P. Nash: SH2 domains, interaction modules and cellular wiring. *Trends Cell Biol* 11(12), 504-511 (2001)

57. Songyang, Z., S.E. Shoelson, M. Chaudhuri, G. Gish, T. Roberts, S. Ratnofsky, R.J. Lechleider, B.G. Neel, R.B. Birge, J.E. Fajardo, M.M. Chou, H. Hanafusa, B. Schaffhausen, and L.C. Cantley: SH2 domains recognize specific phosphopeptide sequences. *Cell* 72, 767-778 (1993)

58. Pawson, T.: Protein modules and signaling networks. *Nature* 373, 573-580. (1995)

59. Stein, E.G., R. Ghirlando, and S.R. Hubbard: Structural basis for dimerization of the Grb10 SH2 domain: implications for ligand specificity. *J. Biol. Chem.* 278, 13257-13264 (2003)

60. Moutossamy, S., F. Renaudie, F. Lago, P.A. Kelly, and J. Finidori: Grb10 identified as a potential regulator of growth hormone (GH) signaling by cloning of GH receptor target proteins. *J. Biol. Chem.* 273, 15906-15912. (1998)

61. Stein, E., D.P. Cerretti, and T.O. Daniel: Ligand activation of ELK receptor tyrosine kinase promotes its association with Grb10 and Grb2 in vascular endothelial cells. *J. Biol. Chem.* 271(38), 23588-23593. (1996)

62. Morrione, A., P. Plant, B. Valentinis, O. Staub, S. Kumar, D. Rotin, and R. Baserga: mGrb10 Interacts with Nedd4. *J. Biol. Chem.* 274, 24094-24099 (1999)

63. He, W., D.W. Rose, J.M. Olefsky, and T.A. Gustafson: Grb10 interacts differentially with the insulin receptor, insulin-like growth factor I receptor, and epidermal growth factor receptor via the Grb10 Src homology 2 (SH2) domain and a second novel domain located between the Pleckstrin homology and SH2 domains. *J. Biol. Chem.* 273, 6860-6867. (1998)

64. Kumar, S., Y. Tomooka, and M. Noda: Identification of a set of genes with developmentally down-regulated expression in the mouse brain. *Biochem Biophys Res Commun* 185(3), 1155-1161 (1992)

65. Kumar, S., K.F. Harvey, M. Kinoshita, N.G. Copeland, M. Noda, and N.A. Jenkins: cDNA cloning, expression analysis, and mapping of the mouse Nedd4 gene. *Genomics* 40(3), 435-443 (1997)

66. Stein, E.G., T.A. Gustafson, and S.R. Hubbard: The BPS domain of Grb10 inhibits the catalytic activity of the insulin and IGF1 receptors. *FEBS Lett* 493, 106-111. (2001)

67. Durick, K., R.-Y. Wu, G.N. Gill, and S.S. Taylor: Mitogenic signaling by Ret/ptc2 requires association with Enigma via a LIM domain. *J. Biol. Chem.* 271, 12691-12694. (1996)

68. Morrione, A., B. Valentinis, S. Li, J.Y.T. Ooi, B. Margolis, and R. Baserga: Grb10: A new substrate of the insulin-like growth factor I receptor. *Cancer Res.* 56, 3165-3167. (1996)

69. Hubbard, S.R.: Crystal structure of the activated insulin receptor tyrosine kinase in complex with peptide substrate and ATP analog. *Embo J* 16(18), 5572-5581 (1997)

70. Favelyukis, S., J.H. Till, S.R. Hubbard, and W.T. Miller: Structure and autoregulation of the insulin-like growth factor 1 receptor kinase. *Nat Struct Biol* 8(12), 1058-1063 (2001)

71. Daughaday, W.H. and P. Rotwein: Insulin-like growth factors I and II. Peptide, messenger ribonucleic acid and gene structures, serum, and tissue concentrations. *Endocr Rev* 10(1), 68-91 (1989)

72. Kahn, C.R.: Banting Lecture. Insulin action, diabetogenes, and the cause of type II diabetes. *Diabetes* 43(8), 1066-1084 (1994)

73. Jones, J.I. and D.R. Clemmons: Insulin-like growth factors and their binding proteins: biological actions. *Endocr Rev* 16, 3-34. (1995)

74. LeRoith, D., H. Werner, D. Beitner-Johnson, and C.T. Roberts, Jr.: Molecular and cellular aspects of the insulin-like growth factor I receptor. *Endocr Rev* 16, 143-163. (1995)

75. Blakesley, V.A., A. Scrimgeour, D. Esposito, and D. Le Roith: Signaling via the insulin-like growth factor-I receptor: does it differ from insulin receptor signaling? *Cytokine Growth Factor Rev* 7(2), 153-159 (1996)

76. Bereziat, V., A. Kasus-Jacobi, D. Perdereau, B. Cariou, J. Girard, and A.F. Burnol: Inhibition of insulin receptor catalytic activity by the molecular adapter Grb14. *J Biol Chem* 277(7), 4845-4852 (2002)

77. Wick, K.R., E.D. Werner, P. Langlais, F.J. Ramos, L.Q. Dong, S.E. Shoelson, and F. Liu: Grb10 inhibits insulin-stimulated IRS/PI 3-Kinase/Akt Signaling pathway by disrupting the association of IRS-1/IRS-2 with the insulin receptor. *J. Biol. Chem.* 278, 8460-8467 (2003)

78. Kullander, K. and R. Klein: Mechanisms and functions of Eph and ephrin signalling. *Nat Rev Mol Cell Biol* 3(7), 475-486 (2002)

79. van der Geer, P., T. Hunter, and R.A. Lindberg: Receptor protein-tyrosine kinases and their signal transduction pathways. *Annu Rev Cell Biol* 10, 251-337 (1994)

80. Manie, S., M. Santoro, A. Fusco, and M. Billaud: The RET receptor: function in development and dysfunction in congenital malformation. *Trends Genet* 17(10), 580-589 (2001)

81. Pandey, A., X. Liu, J.E. Dixon, P.P. Di Fiore, and V.M. Dixit: Direct association between the Ret receptor tyrosine kinase and the Src homology 2-containing adaptor protein Grb7. *J. Biol. Chem.* 271(18), 10607-10610. (1996)

82. Mano, H., K. Ohya, A. Miyazato, Y. Yamashita, W. Ogawa, J. Inazawa, U. Ikeda, K. Shimada, Hatake K, M. Kasuga, K. Ozawa, and S. Kajigaya: Grb10/GrbIR as an in vivo substrate of Tec tyrosine kinase. *Genes Cells* 3, 431-441. (1998)

83. Jahn, T., P. Seipel, S. Urschel, C. Peschel, and J. Duyster: Role for the adaptor protein grb10 in the activation of akt. *Mol Cell Biol* 22, 979-991. (2002)

84. Chan, L.C., K.K. Karhi, S.I. Rayter, N. Heisterkamp, S. Eridani, R. Powles, S.D. Lawler, J. Groffen, J.G. Foulkes, M.F. Greaves, and et al.: A novel abl protein expressed in Philadelphia chromosome positive acute lymphoblastic leukaemia. *Nature* 325(6105), 635-637 (1987)

85. Advani, A.S. and A.M. Pendergast: Bcr-Abl variants: biological and clinical aspects. *Leuk Res* 26(8), 713-720 (2002)

86. Daley, G.Q. and D. Baltimore: Transformation of an interleukin 3-dependent hematopoietic cell line by the chronic myelogenous leukemia-specific P210bcr/abl protein. *Proc Natl Acad Sci U S A* 85(23), 9312-9316 (1988)

87. Pillai, S. and S.T. Moran: Tec kinase pathways in lymphocyte development and transformation. *Biochim Biophys Acta* 1602(2), 162-167 (2002)

88. Nantel, A., M. Huber, and D.Y. Thomas: Localization of endogenous Grb10 to the mitochondria and its interaction with the mitochondrial-associated Raf-1 pool. *J Biol Chem* 274(50), 35719-35724. (1999)

89. Linnekin, D.: Early signaling pathways activated by c-Kit in hematopoietic cells. *Int J Biochem Cell Biol* 31(10), 1053-1074 (1999)

90. Carter-Su, C., A.P. King, L.S. Argetsinger, L.S. Smit, J. Vanderkuur, and G.S. Campbell: Signalling pathway of GH. *Endocr J* 43 Suppl, S65-70 (1996)

91. Harvey, K.F. and S. Kumar: Nedd4-like proteins: an emerging family of ubiquitin-protein ligases implicated in diverse cellular functions. *Trends Cell Biol* 9(5), 166-169 (1999)

92. Yeh, E.T., L. Gong, and T. Kamitani: Ubiquitin-like proteins: new wines in new bottles. *Gene* 248(1-2), 1-14 (2000)

93. Ciechanover, A.: The ubiquitin-proteasome proteolytic pathway. *Cell* 79(1), 13-21 (1994)

94. Cook, K.K. and D.A. Fadool: Two adaptor proteins differentially modulate the phosphorylation and biophysics of Kv1.3 ion channel by SRC kinase. *J Biol Chem* 277(15), 13268-13280 (2002)

95. Kues, W.A. and F. Wunder: Heterogeneous Expression Patterns of Mammalian Potassium Channel Genes in Developing and Adult Rat Brain. *Eur J Neurosci* 4(12), 1296-1308 (1992)

96. Hille, B., *Ion Channels of Excitable Membranes*. 3 ed: Sinauer Associates. 722 (2001)

97. Kim, E., M. Niethammer, A. Rothschild, Y.N. Jan, and M. Sheng: Clustering of Shaker-type K+ channels by interaction with a family of membrane-associated guanylate kinases. *Nature* 378(6552), 85-88 (1995)

98. Holmes, T.C., D.A. Fadool, R. Ren, and I.B. Levitan: Association of Src tyrosine kinase with a human potassium channel mediated by SH3 domain. *Science* 274(5295), 2089-2091 (1996)

99. Tiffany, A.M., L.N. Manganas, E. Kim, Y.P. Hsueh, M. Sheng, and J.S. Trimmer: PSD-95 and SAP97 exhibit distinct mechanisms for regulating K(+) channel surface expression and clustering. *J Cell Biol* 148(1), 147-158 (2000)

100. Joiner, W.J., R. Khanna, L.C. Schlichter, and L.K. Kaczmarek: Calmodulin regulates assembly and trafficking of SK4/IK1 Ca2+-activated K+ channels. *J Biol Chem* 276(41), 37980-37985 (2001)

101. Langlais, P., L.Q. Dong, D. Hu, and F. Liu: Identification of Grb10 as a direct substrate for members of the Src tyrosine kinase family. *Oncogene* 19, 2895-2903 (2000)

102. Hannon, G.J.: RNA interference. *Nature* 418(6894), 244-251 (2002)

103. Tanaka, S., M. Mori, T. Akiyoshi, Y. Tanaka, K. Mafune, J.R. Wands, and K. Sugimachi: A novel variant of human Grb7 is associated with invasive esophageal carcinoma. *J Clin Invest* 102(4), 821-827 (1998)

Send correspondence to: Feng Liu, Department of Pharmacology, University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Drive, Tx 78229. Tel: 210-567-3097, Fax: 210-567-4303. E-mail: liuf@uthscsa.edu