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GRB10 EXCEEDING THE BOUNDARIES OF A COMMON SIGNALING ADAPTER

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1. ABSTRACT

*G*rowth factor *r*eceptor *b*inding protein 10 (Grb10) has been identified as a cellular partner of a number of receptor tyrosine kinases and other signaling mediators, compatible with multiple roles in mitogenic, metabolic, and embryogenic signaling that are also supported by the tissue distribution of Grb10. In particular, a role has been implicated in the regulation of PI 3-kinase signaling downstream of the insulin receptor. At least seven alternative splice variants have been identified within the Grb10 gene, a proposed candidate for some types of human Silver-Russell syndrome. Located on chromosome 7 (human) or 11 (mouse) the gene is oppositely imprinted in both species. Grb10 isoforms are members of a super family of signaling mediators that includes Grb7, Grb14, and *Caenorhabditis elegans* MIG-10. All mammalian members of this family share a domain structure which is represented by N-terminal (proline) Pro-rich sequences, a homology domain with MIG-10 (GM) which includes a Ras-associating (RA)-like domain, a pleckstrin homology region (PH), a C-terminal Src homology 2 (SH2) domain, and a receptor binding domain located *b*etween the *P*H and the *S*H2 domains termed BPS. Various Grb10 isoforms have been identified as cellular partners of the insulin receptor (IR) and insulin-like growth factor-I (IGF-I) receptor that provide the best-established regulators of Grb10 signaling. A regulatory role of Grb10 has been established in the respective metabolic and mitogenic responses by numerous lines of experimental evidence. However, the specific contribution of Grb10 was found to be highly dependent on the cellular context including the balance of other signaling mediators that define whether increased Grb10 levels will enhance or restrain a given response. This is supported by observations with super family members Grb7 and Grb14 that may engage in competitive and redundant mechanisms when compared to Grb10. Grb10 gene disruption in the mouse results in

embryonal and placental overgrowth. The underlying molecular mechanisms and their interpretation remain open until a more comprehensive analysis will be available which includes the contribution of the Grb7 and Grb14 super family members. From a physiologic perspective at the cellular level increased levels of Grb10 have been shown to stimulate insulin metabolic action or mitogenic growth factor responses whereas peptide mimetics representing individual Grb10 domains were found to act oppositely by inhibiting the respective cellular response. In an alternative experimental context increased cellular levels of Grb10 have repeatedly been shown to inhibit cellular responses and signaling mechanisms. This has been most specifically observed at the level of molecular interactions *in vitro*. How the various observations relate to the physiologic role of cellular Grb10 remains to be established, also in the context of possible cross-talk to Grb14 and Grb7 signaling. Based on its interactions with a number of signaling mediators including protein kinases, adapters, and enzymes such as a ubiquitin ligase, Grb10 may act as a signaling hub to integrate multiple incoming signals and as a molecular scaffold to help assemble signaling complexes. The specific contribution of Grb10 in a signaling complex may depend on the local stochiometric balance of associating mediators, including the ratio of competing signaling proteins. In this context a constant cellular level of Grb10 may enhance or restrain a specific signaling mechanism depending on the local distribution and balance of specific Grb10 signaling partners. This concept is compatible with the diverse experimental observations on Grb10 function and emphasizes the importance of the specific cellular context to define the consequences of local changes in Grb10 distribution. Thus, to think of Grb10 as either a positive or negative signaling mediator will be inadequate in reflecting the complexity that underlies the final output of the Grb10 signal.

2. INTRODUCTION

2.1. Signaling partners suggest diverse cellular roles of Grb10

Growth factor receptor binding protein 10 (Grb10) has been identified as a cellular partner of a number of receptor tyrosine kinases and other signaling mediators compatible with a role in a variety of signaling mechanisms (see adjacent review in 1). Initially, Grb10 was discovered as a partner of epidermal growth factor (EGF) receptor (2), however, a physiologic role of Grb10 in EGF action has not been established. Grb10 interacts with the Ret receptor tyrosine kinase that has been implicated in the development of the enteric nervous, endocrine, and renal systems, as well as in papillary thyroid cancer (3, 4). Grb10 associates with the insulin receptor (IR) (5, 6, 7) and the insulin-like growth factor-I (IGF-I) receptor (IGF-IR) (8, 9, 10, 11) that carry out important metabolic and mitogenic functions. A preference was observed for IR in a direct comparison (12). Grb10 has been identified as a target of the Eph-related receptor tyrosine kinase ELK which is involved in axonal guidance, neuronal bundling, and angiogenesis (13) and as a target of growth hormone receptor via Janus kinase Jak2 (14). The Grb10 SH2 domain has been shown to associate with activated platelet-derived growth factor (PDGF) receptor beta (PDGFR), hepatocyte growth factor receptor (c-Met), and fibroblast growth factor receptor, but not with EGF receptor or nerve growth factor receptor TrkA in a direct comparison (15). Grb10 interacts with ubiquitin ligase Nedd 4 (16), the oncogenic tyrosine kinase Bcr-Abl (7), tyrosine kinase Tec (18, 19), MEK and Raf-1 (20), Akt and c-Kit (21), the regulatory subunit p85 of phosphatidylinositol (PI) 3-kinase (22), and GIGYF1 and GIGYF2 (23). The functional implications of some of these interactions and emerging concepts of Grb10 signaling have been addressed and are highlighted in a model at the end of this review.

2.2. Grb10 SH2 domain provides key interface for phosphotyrosine interactions

Various signaling mediators have been reported to bind to the SH2 domain of Grb10 including the receptor tyrosine kinases for EGF, insulin, IGF-I, PDGF, fibroblast growth factor, and hepatocyte growth factor (2, 5, 6, 8, 12, 15, 24) as well as proto-oncogene product Ret, a putative receptor (3). The SH2 domain plays a differential role in the interaction of Grb10 with various receptors. In competition experiments with Grb10 SH2 domain peptide mimetics the association of cellular Grb10 with PDGF receptor was fully inhibited, the association with the insulin receptor partially, whereas the association with the IGF-I receptor was not found significantly affected (15). These observations support the role of a second Grb10 domain, the BPS domain in the interaction with the insulin and IGF-I receptors and suggest against a major role of the SH2 domain in the association with the IGF-I receptor in line with a proposed model (25). They suggest against a role of other Grb10 domains in addition to the SH2 domain in the association with the PDGF receptor (15). Other tyrosine kinase binding partners of Grb10 include ELK1 (13) and transforming protein Bcr-Abl (17). The Grb10 SH2 domain interacts with phosphorylated tyrosine residues of growth hormone receptor that lacks intrinsic tyrosine kinase activity (14). The Grb10 SH2 domain has been reported to associate in a phosphotyrosine-independent manner with Raf1, MEK1, or Nedd4 (neuronal precursor cell-expressed developmentally down-regulated 4) (16, 20). Such an association is supported by the structural analysis of the Grb10 SH2 domain (26). At a more physiologic level, a Grb10 SH2 domain peptide mimetic significantly interfered with insulin-, IGF-I-, and PDGF-mediated mitogenesis as measured in NIH 3T3 or baby hamster kidney (BHK) fibroblasts by DNA synthesis (15) or cell proliferation (Deng and Riedel, unpublished data). Similarly, the Grb10 SH2 domain peptide mimetic substantially interfered with key insulin-stimulated metabolic responses including lipogenesis, glycogen synthesis, glucose and amino acid transport and with key enzyme activities in the involved insulin signaling pathway including PI 3-kinase, PKB/Akt, glycogen synthase, and glycogen synthase kinase (22). The data indicate a role of the SH2 domain in all regulated mechanisms despite the fact that the Grb10 BPS domain has been implicated as an additional interface in the Grb10 association with IR and as the key interface in the association with IGF-IR (15, 25). In combination, the Grb10 SH2 domain plays a critical role

in key intermolecular and intra-molecular interactions in the context of Grb10 signaling.

2.3. Seven alternatively-spliced products of the Grb10 gene re-define variant nomenclature

At least seven splice variants have been identified in the human and mouse Grb10 gene (Figure 1), located on chromosome 7 (human) or 11 (mouse) (27, 28), a candidate gene for human Silver-Russell syndrome based on its specific chromosomal location (29, 30, 31). An emerging consensus nomenclature (Figure 1) has been adopted throughout this review that identifies each Grb10 variant in the order of discovery by greek letter (20, 32, 33) beginning with mouse Grb10 alpha. This has been defined in the legend to Figure 1 (see also *http://cbr-rbc.nrc-cnrc.gc.ca/thomaslab/grb7.html* for more information about the super family and http://www3.ncbi.nlm.nih.gov:80/htbin-post/Omin/dispmim?601523). Three mouse Grb10 variants have been reported, all sharing high sequence homology with human Grb10. Two carry overlapping inserts in the proline-rich N-terminal region of 79 aa (mouse Grb10 alpha; 2) or 55 aa (mouse Grb10 delta; 12) in length and one lacks this insert or any other major change (mouse Grb10 eta; 34) when compared to human Grb10 gamma (7, 24). Up to this date in reports on mouse Grb10, apparently all cDNA expression studies (15, 22, 35, 36, 37) are based on the mouse delta variant (12) which is not always properly identified in the published articles due to lack of clarification by the donor of the mouse Grb10 cDNA. Experiments referring to cDNA expression of mouse Grb10 alpha actually represent expression of the mouse delta variant. Potential functional differences between mouse and/or human variants remain unknown.

Human sequences have originally been termed Grb-IR or hGrb10 alpha, Grb10/IR-SV1 or hGrb-IRbeta/Grb-10, hGrb-10 gamma and delta (5, 7, 11, 24) in the absence of a consistent nomenclature. In this review as shown in Figure 1 according to the new nomenclature the first discovered mouse Grb10 variant (2) is represented as mGrb10 alpha; Grb-IR (5) as hGrb10 beta; Grb10/IR-SV1 (24) and hGrb-IRbeta/Grb10 (7) as hGrb10 gamma; a second mouse Grb10 variant (12) as mGrb10 delta; hGrb10 delta (11, 38) or KIAA0207 as hGrb10 epsilon; hGrb10 gamma (11) as hGrb10 zeta, and a third mouse Grb10 variant sequence (34) as mGrb10 eta. Human Grb10 gamma represents the closest match to mouse variants, in particular to mouse Grb10 eta where any differences are limited to point mutations (Figure 1B). In comparison, human Grb10 variants beta and zeta share an N-terminal extension of 58 amino acids whereas human Grb10 epsilon carries a unique N-terminal extension of 52 amino acids. Human Grb10 beta carries an additional internal deletion of 46 amino acids that truncates the PH region (Figure 1).

2.4. Domain structure and tissue distribution define super family of signaling mediators with overlapping features

Based on structural similarities the Grb10 isoforms are members of the Grb7/10/14 super family of signaling mediators which include Grb7, Grb14, and *Caenorhabditis elegans* MIG-10 and their splice variants (32, 38). Grb7/10/14 family members (32, 33,39) share (proline) Pro-rich sequences at the N-terminus, a region termed GM (*G*rb/*M*ig) which contains a Ras-associating (RA)-like domain (40) and is followed by a pleckstrin homology (PH) region, and a receptor binding region located *b*etween the *P*H and *S*H2 domains termed BPS (or IPS) (25, 41). Super family members carry an SH2 domain at the C-terminus except for MIG-10 that contains a Pro-rich sequence instead (42). Both the SH2 and BPS domains have been implicated in the association with receptor tyrosine kinases (15, 25, 41). Northern analysis indicates an overlapping but distinct expression profile of Grb7/10/14 super family members in human tissues but none have been found in thymus and peripheral blood lymphocytes (32). Mouse Grb10 expression has been reported based on Northern analysis in heart, kidney, brain, and lung (2) and independently, with a consistent message size of 6 kb in heart, brain, skeletal muscle, and testis (Figure 2), whereas consistently expression was not observed in spleen and marginally in liver. In an expanded analysis by mRNA in situ hybridization and immunohistochemistry Grb10 expression in the mouse has been detected in various muscles, brain, liver, lung, long bones, and also in the adrenal gland and pancreatic bud (43). High expression levels of human Grb10 have been observed in pancreas, skeletal muscle, brain and heart (7). Grb7 is highly expressed in pancreas, kidney, placenta, prostate, small intestine (7) and Grb14 in pancreas, kidney, skeletal muscle, liver, heart, ovary, and testis (44). The *Caenorhabditis elegans* gene mig-10 has been implicated to control cell migration in the development of the excretory canal (42).

3. GRB10 IN INSULIN ACTION

3.1. Grb10 domains in the regulation of metabolic and mitogenic responses

At a physiologic level, a role of mouse Grb10 delta has been implicated in the metabolic and mitogenic response to insulin and in the mitogenic responses to the related IGF-I, and to PDGF and other peptide growth factors. Elevated levels of Grb10 were found stimulatory whereas Grb10 domain specific peptide mimetics were found to block the respective physiologic response in a dose-dependent fashion, consistent with a dominant-negative role (15). A 16 amino acid proline-rich Grb10 peptide mimetic significantly interfered with insulin- and IGF-I-, but not with PDGF-mediated mitogenesis as measured in NIH 3T3 or baby hamster kidney (BHK) fibroblasts by DNA synthesis or cell proliferation. In a direct comparison, a Grb10 SH2 domain peptide mimetic blocked, and over-expression of complete mouse Grb10 delta 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 (15). Similarly, in differentiated, cultured mouse 3T3-L1 adipocytes the proline-rich or SH2 domain peptide mimetics substantially interfered with key insulin-stimulated metabolic responses including lipogenesis, glycogen synthesis, glucose and amino acid transport all of which were stimulated by

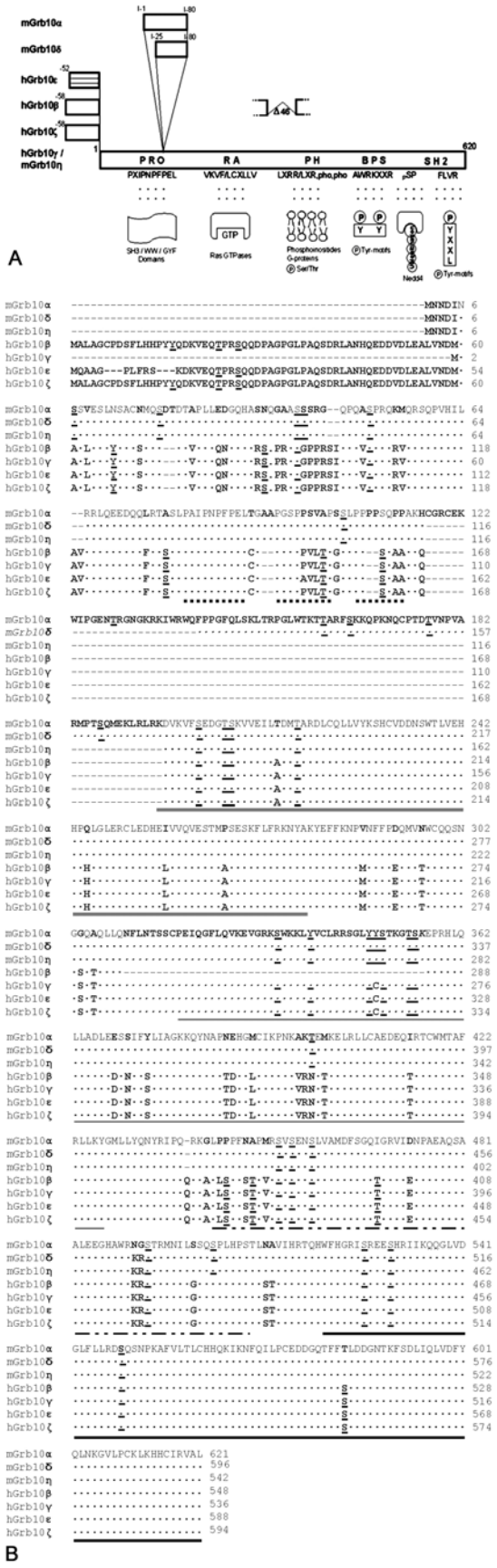

Figure 1. Comparison of Grb10 alternative splice variants. Amino acid sequences of mouse Grb10 variants alpha (α), delta (δ), eta (η), and human Grb10 variants beta (β), gamma (γ), epsilon (ε), zeta (ζ) have been aligned for maximum similarity and Pro-rich (PRO), Ras-associating-like (RA), pleckstrin homology (PH), BPS (*b*etween the *P*H and *S*H2 domains), and Src homology 2 (SH2) domains (including Nedd4 binding motif pSP) have been indicated. An emerging consensus nomenclature has been adopted throughout this review that identifies each Grb10 variant in the order of discovery by greek letter (20, 32, 33) beginning with mouse Grb10 alpha (see also *http://cbr-rbc.nrc-cnrc.gc.ca/thomaslab/grb7.html* for more information about the super-family and http://www3.ncbi.nlm.nih.gov:80/htbin-post/Omin/dispmim?601523). Mouse Grb10 (2) is represented as mGrb10 alpha; Grb-IR (5) as hGrb10 beta; Grb10/IR-SV1 (24) and hGrb-IRbeta/Grb10 (7) as hGrb10 gamma; a second mouse Grb10 variant (12) as mGrb10 delta; hGrb10 delta (11, 38) or KIAA0207 as hGrb10 epsilon; hGrb10 gamma (11) as hGrb10 zeta, and a third mouse Grb10 variant sequence (34) as mGrb10 eta. GenBank™ accession numbers: mGrb10 alpha (*U18996*), mGrb10 delta (*AF022072*), mGrb10 eta (*AAH16111*), hGrb10 beta (*U34355*), hGrb10 gamma (*U66065* / *U69276*), hGrb10 epsilon (*D86962*), hGrb10 zeta (*AF000017* / *AF001534*). **A.** Amino acids shown below each domain represent a Grb10 sequence motif predicted to be critical in the association with a target domain (identified by type) of independent signaling mediators shown underneath. Amino acid sequences are represented by standard letter code, phosphoserine representing several putative phosphorylation sites by (pS), positions which permit the presence of any amino acid by (X), and hydrophobic amino acids by (pho). Intermolecular interactions are represented by pairs of dots. Human Grb10 gamma (γ) and mouse Grb10 eta (η) which share the most simple domain structure have been used as a reference. For other variants only changes in the domain structure have been shown. Boxes represent amino acid sequences as indicated by numbers. The unique N-terminal sequencesof hGrb10ε is represented by a striped box. The deletion of 46 amino acids in hGrb10β between aa 282 and aa 283 corresponds to aa 225 to 270 of hGrb10γ and aa 311 to 356 of mGrb10α. Insert (I-1 to I-80), corresponds to aa 117 to 196 of mGrb10α and insert (I-25 to I-80), corresponds to aa 117 to 171 of mGrb10δ. B. Complete predicted primary structures of mouse and human Grb10 variants have been aligned for maximum similarity. The mouse Grb10 alpha sequence serves as a reference shown at the top and identical amino acids in other variants are represented by dots whereas amino acids that are not conserved have been individually identified by bold letters. Gaps shown as (-) were introduced where necessary to align sequences for maximum similarity. Potential Ser, Thr, or Tyr phosphorylation target residues (105) are underlined. Specific amino acid sequence motifs and domains have been underlined (7, 23, 25, 40, 44): Pro-rich (*heavy squared dots*), RA (*double line*), PH (*thin line*), BPS (*broken hatched and dotted line*), and SH2 (*heavy line*). Amino acid numbers are shown on the right starting with (1) at the initiator methionine (M) for each variant.

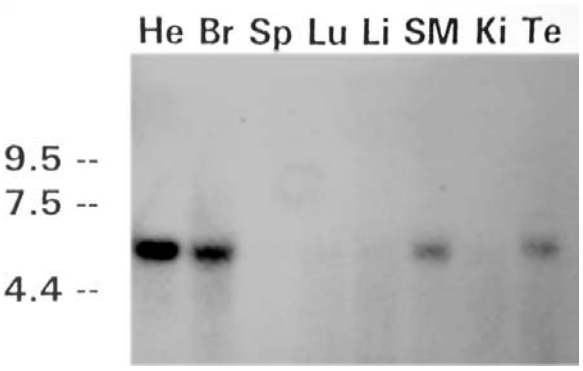

Figure 2. Northern analysis of Grb10 expression in mouse tissues. Various tissues including heart (He), brain (Br), spleen (Sp), lung (Lu), liver (Li), skeletal muscle (SM), kidney (Ki), and testis (Te) have been compared. Poly A^+ mouse mRNA separated by gel electrophoresis and transferred to a nylon membrane was hybridized with a ^{32}P-labeled cDNA fragment encoding the conserved C-terminus of the Grb10 variant family. Size markers are indicated in kb next to the autoradiograph.

increased levels of mouse Grb10 delta (22). Insulin activation of key enzymes in the involved insulin signaling pathway including PI 3-kinase, PKB/Akt, glycogen synthase, and glycogen synthase kinase was substantially inhibited by the proline-rich or SH2 domain peptides whereas the respective enzyme activities were stimulated by increased levels of mouse Grb10 delta. In contrast, insulin activation of key enzymes in alternative signaling pathways such as p38 MAP kinase, p70 S6 kinase, and c-Cbl remained unaffected by the proline-rich or SH2 domain peptides or by increased levels of mouse Grb10 delta. Similarly, insulin-stimulated phosphorylation of the insulin receptor, IRS-1, or IRS-2 remained unaffected by either peptide or by increased levels of mouse Grb10 delta (22). At a physiologic level, these combined data support a stimulatory role of mouse Grb10 delta in specific key metabolic and mitogenic signaling pathways in several alternative host cells. The data 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. A stimulatory role was also supported by the observed inhibition of insulin and IGF-I-stimulated mitogenesis in response to microinjection of a putative dominant-negative human Grb10 gamma peptide mimetic of the BPS and SH2 domains (24). A peptide mimetic of the Grb10 BPS domain inhibited substrate phosphorylation of activated IR and the catalytic activity of activated IGF-IR *in vitro* (45).

3.2. Interference with molecular interactions and physiologic responses

Using a lytic, recombinant adenovirus over-expression system highly elevated levels of mouse Grb10 delta (20-fold over basal cellular levels) in mouse 3T3-L1 adipocytes inhibited insulin-stimulated glucose uptake by 50% whereas a mouse Grb10 delta mutant carrying the BPS and SH2 domains but lacking the N-terminal region did not affect glucose uptake under any conditions (37). At high over-expression levels both Grb10 constructs partially interfered with insulin-stimulated IRS-1/2, Akt, Shc, ERK1/2, and c-Cbl phosphorylation but not with IR phosphorylation (37). Key differences to the previous study (22) lie in the 20-fold Grb10 over-expression levels and in the lytic adenoviral expression system that could possibly impact on cellular Grb10 function (as discussed below). Reduced insulin-dependent Tyr phosphorylation of IRS-1, GAP-associated protein p60, and diminished activation of PI 3-kinase had earlier been reported in response to human Grb10 beta over-expression in CHO cells over-expressing IR, an environment which does not allow the evaluation of a native metabolic insulin response (5). The phosphorylation of specific mediators of insulin action was blocked by over-expression of human Grb10 zeta either in CHO cells over-expressing IR or by adenovirus-mediated high-level expression of human Grb10 zeta in mouse 3T3-L1adipocytes (46). In these experiments partial interference was reported with IRS-1, IRS-2, and Akt phosphorylation but not with IR autophosphorylation. The physiologic role of Grb10 was not addressed. Yeast-tri-hybrid studies implicated an interfering role of human Grb10 zeta that required its SH2 domain in the association of IRS proteins with IR. Grb10 was proposed to physically block access of IRS proteins to IR which would explain the reported inhibitory effects on downstream mediators of insulin action (46). In contrast, in response to over-expression of human Grb10 zeta in primary rat hepatocytes with a lytic recombinant adenovirus, some reduction of IR autophosphorylation, glycogen synthase activity, and glycogen synthesis was reported and proposed to inhibit an unidentified insulin signaling pathway leading to glycogen synthesis in the liver (47). Surprisingly, insulin-stimulated IRS-1 phosphorylation, PI 3-kinase activation, Akt/PKB activity, GSK3 activity, and ERK1/2 MAP kinase activity were reported unchanged (47).

It would be tempting to explain the reported experimental differences with the distinct employed Grb10 isoforms. Direct functional comparisons of Grb10 isoforms from different species have not yet been reported. However, in an initial comparison of human Grb10 beta, human Grb10 zeta, and mouse Grb10 delta in mouse L6 cells by cDNA expression, all isoforms comparably stimulated insulin-mediated metabolic responses including glycogen synthesis (Figure 3) or glucose uptake (Xu and Riedel, unpublished data). It appears, that the experimental host cell system (insulin-responsive versus fibroblast over-expressing IR), the employed mechanism of Grb10 expression (cell membrane-permeable peptides or cDNA expression versus lytic, high-level recombinant adenovirus infection), and the measured endpoint (*in vivo* physiologic response versus *in vitro* molecular association or activation such as phosphorylation) have a major impact on the experimental outcome and interpretation of the respective result. In the context of the employed lytic recombinant adenovirus Grb10 expression system it should be noted that tumorigenesis observed in Adenovirus 12-transformed cells correlates with an induction of cellular Grb10 expression when compared to non-tumorigenic cells transformed with Adenovirus 5 (48). The lytic adenovirus life cycle has a major impact on cellular signaling pathways that may cross-talk to Grb10-mediated signaling mechanisms and alter the normal Grb10 response.

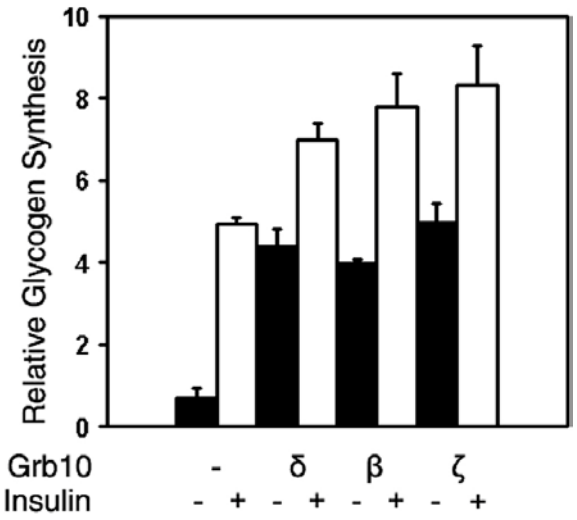

Figure 3. Comparison of mouse and human Grb10 variant regulation of insulin-mediated glycogen synthesis in mouse L6 cells. After transfection with mouse Grb10 delta (δ), human Grb10 beta (β), human Grb10 zeta (ζ), or with control plasmid (-) cells were incubated for 30 min. in the presence or absence of 100 nM insulin. The incorporation of [U-^{14}C]-D glucose into glycogen was quantified by liquid scintillation spectroscopy. The average of several experiments each with multiple data points has been presented including the experimental error.

4. GRB10 IN IGF-I ACTION

4.1. Role in IGF-IR degradation and interference with IGF-IR transformation

Mouse Grb10 delta over-expression in human IGF-IR-transformed (but not in functional IGF-IR-deficient) mouse fibroblasts inhibited IGF-I-mediated cell proliferation in a Grb10 dose-dependent fashion (35). In comparison, insulin-mediated proliferation in IR over-expressing cells remained unaffected by Grb10 suggesting a significant difference between the two receptors with respect to the Grb10 signal (35, 49). The observed interference was found dependent on complete human IGF-IR and was abolished by C-terminal truncation of 108 amino acids, supporting the predicted association of Grb10 with a phosphotyrosine in the respective C-terminal region of IGF-1R (8, 15). Mouse Nedd4-1 (*n*euronal precursor cell-*e*xpressed *d*evelopmentally *d*own-regulated 4) was identified as a Grb10-associated protein in a two-hybrid screen (16). Nedd4 family members are ubiquitin-ligases (E3) that carry out the substrate-specific and final step of the ubiquitination pathway to label proteins for rapid degradation (50). Association of the Grb10 SH2 domain with the Nedd4 C2 domain was constitutive, independent of Ca^{2+} and phosphotyrosine but did not result in detectable Grb10 ubiquitination (16). IGF-I induced binding of the Grb10 BPS domain to activated IGF-IR, resulted in close proximity between Nedd4 and the receptor, and led to ubiquitination and subsequent degradation of IGF-IR (36). This finding implicates a role of the Nedd4 family of E3 ubiquitin ligases in the regulation of internalization and stability of receptor tyrosine kinases.

An intriguing possibility to explain alternative responses to increased cellular levels of Grb10 may lie in the duration of the respective signal. Stimulatory effects on mitogenesis have typically been observed within one to several days after Grb10 expression or after treatment with dominant-negative peptides resulting in transient changes in Grb10 activity (15, 24) whereas inhibitory mitogenic effects have been observed in Grb10 stably expressing cell lines at constant Grb10 levels over periods of many days (43). It is conceivable that at different time points the Grb10 signal may result in opposing mitogenic responses of which the inhibitory role prevails over long durations compatible with the observations in the mouse embryo (43).

4.2. Proline-rich sequences provide interface for mediators GIGYF1 and GIGYF2

Members of the Grb7/10/14 super family share an extended proline-rich region at the N-terminus (Figure 1) that may interact with Src-homology 3 (SH3), WW (small globular domains rich in tryptophan), or GYF domains of other signaling mediators (51, 52). The SH3 domain of tyrosine kinase proto-oncogene product c-Abl interacts with rat Grb10 *in vitro* in competition with a proline-rich peptide mimetic (7). However, this interaction could not be confirmed by co-immunoprecipitation experiments from cell extracts (17) whereas the association between Grb10 and oncogene product Bcr-Abl appears to rely on the Grb10 SH2 domain instead. A GYF domain (52) represented by a 17 amino acid motif in T lymphocyte CD2 binding protein 2 (CD2BP2) was shown to mediate association with two proline-rich boxes of CD2 (53). Two cellular partners of Grb10, carrying a GYF domain have been identified and termed GIGYF1 and GIGYF2 (23). A 17 amino acid GYF homology sequence in either protein mediates binding to tandem proline-rich regions near the N-terminus of Grb10. GIGYF1 binds to Grb10 which is further stimulated by IGF-I, and both mediators associate with IGF-IR, albeit GIGYF1 only transiently. GIGYF1 may cooperatively enhance the association between Grb10 and IGF-IR. Over-expression of the Grb10 binding region of GYGIF1 enhances IGF-IR-stimulated tyrosine phosphorylation in a putatively cooperative mechanism with Grb10 to regulate receptor signaling (23). The molecular mechanism remains to be elucidated, however, it is conceivable that GIGYF1 and/or GIGYF2 participate in signaling mechanisms that have been described at a physiologic level and involve the N-terminal proline-rich region of Grb10. A 16 amino acid proline-rich N-terminal Grb10 peptide mimetic significantly interfered with insulin- and IGF-I-, but not with PDGF-mediated mitogenesis as measured in NIH 3T3 or baby hamster kidney (BHK) fibroblasts by DNA synthesis (15) or cell proliferation (Deng and Riedel, unpublished data). In a direct comparison, an SH2 domain peptide mimetic blocked, all three hormone responses (15). 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 peptides. Similarly, the 16 amino acid proline-rich peptide mimetic substantially interfered with key insulin-stimulated metabolic responses including lipogenesis, glycogen

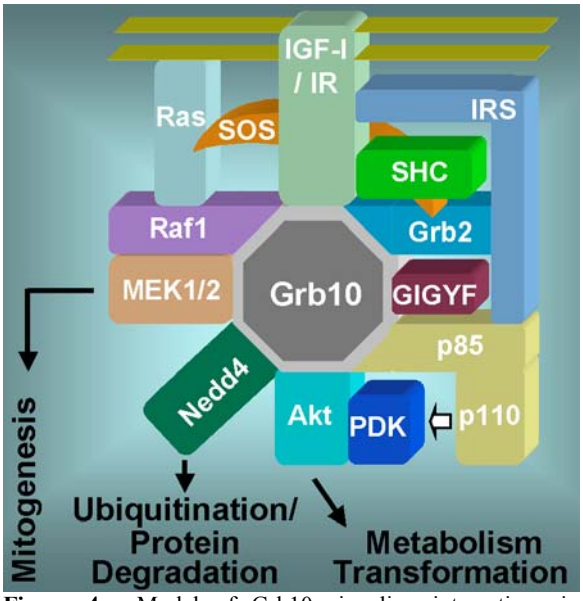

Figure 4. Model of Grb10 signaling interactions in response to IR/IGF-IR activation. Grb10 is displayed as an octagon in the center and associated cellular partners are represented by mostly rectangular objects, identified by specific name in white letters. Contact between objects represents a postulated direct cellular association. The mediator Sos is shown as a sickle-shaped object connecting mediators Grb2 and Shc to the distant Ras. The plasma membrane is represented by dual horizontal sheets. Signaling pathways are shown by arrows with physiologic endpoints identified by black letters. Relative sizes and shapes are arbitrary and have been chosen exclusively to optimize the display of putative interactions in two dimensions.

synthesis, glucose and amino acid transport or with insulin activation of specific key enzymes in the involved insulin signaling pathway including PI 3-kinase, PKB/Akt, glycogen synthase, and glycogen synthase kinase (22). The data implicate a role of the Grb10 proline-rich region in selected signaling mechanisms in response to insulin and IGF-I but excluding the mitogenic response to PDGF.

4.3. Super family-specific BPS domain provides interface for IGF-IR and contributes to interface for IR

Two-hybrid system and *in vitro* peptide interaction maps unveiled an additional receptor binding region of approximately 50 amino acids located *b*etween the *P*H and *S*H2 domains, termed BPS (Figure 1). It is highly conserved within, and unique to, the Grb7/10/14 super family (25). A GST-Grb10 fusion protein that interacted more efficiently with IR *in vitro* than the SH2 domain alone suggested the presence of an additional receptor-binding interface (7). Mutation of the critical arginine within the Grb10 SH2 domain FLVES motif only partially interfered with IR binding (25). Similarly, a Grb10 SH2 domain peptide mimetic reduced co-immunoprecipitation of Grb10 with IR only partially and with IGF-IR not detectably (15). Since the mitogenic response to either insulin or IGF-I was inhibited by the Grb10 SH2 domain peptide mimetic, a role of the Grb10 SH2 domain is implicated in an interaction with an unidentified mediator in the IGF-I response (15). An interaction of Nedd4 was demonstrated in a two-hybrid assay with the Grb10 BPS domain but the SH2 domain was implicated in a more important role in this interaction (16). Mutation of the paired tyrosine Y1162/1163 in the IR activation loop to phenylalanine reduced binding between IR and the individual BPS and SH2 domains, indicating that the tyrosine residues contribute to the interaction with both domains. IR autophosphorylation may involve a conformational change within the kinase domain to expose a binding site for the BPS domain. The interaction of the combined BPS and SH2 domains with the phosphorylated IR kinase domain held up to competition with a phosphopeptide containing all three IR autophosphorylation sites (45) suggesting an additional interface between Grb10 and IR (33). The BPS domain plays a critical role in the receptor-specific interaction of Grb10. This may add to the functional complexity of the distinct signaling complexes that are predicted to form in response to alternative peptide hormones. The molecular role of the BPS domain and its apparent limitation to the Grb7/10/14 super family remains to be elucidated.

5. KEY INTERACTIONS SUGGEST MODELS OF GRB10 SIGNALING

5.1. RA-like domain suggests interface with G protein signaling which remains to be demonstrated

A potential link between Grb10 and Ras-like GTP-binding proteins was suggested by the discovery of a Ras-associating (RA)-like domain (Figure 1) in the Grb7/10/14 superfamily (40). Its three dimensional structure has been resolved in Ral-guanine nucleotide exchange factor (Ral-GEF) and found to be similar to that of the Ras-binding domain of c-Raf1 despite limited sequence identity. While the structural conservation of the motif does not necessarily imply the function of binding small GTPases of the Ras superfamily it will be intriguing to experimentally test this possibility. So far despite repeated attempts, an association between any Grb7/10/14 superfamily members and small GTPases has not been observed (54).

5.2. Regulation of PI 3-kinase activity through direct interaction with p85 and through regulation of associated mediators

An important role of Grb10 has been discovered in the regulation of the metabolic insulin response at the level of p85 PI 3-kinase (Figure 4) with which mouse Grb10 interacts directly in a putative link parallel to IRS proteins (22). The physiologic impact of Grb10 on the metabolic insulin response is best explained by its regulation of PI 3-kinase activity that has been demonstrated directly at the catalytic level and in many downstream enzymatic and physiologic responses (22). A regulation of PI 3-kinase activity, albeit negatively, has been observed for human Grb10 beta in a CHO/IR cell over-expression host likely through modulation of IRS-1 activity (5). A compatible response has been observed at high human Grb10 zeta expression levels in 3T3-L1 adipocytes using a lytic recombinant adenovirus system

through negative regulation of IRS-1/2 catalytic activity due to interference with access to IR (46). This modulation extends to downstream components of the PI 3-kinase pathway such as Akt whereas modulation of PI 3-kinase has not been shown directly. Grb10 regulation of Akt has also been suggested by a direct and constitutive association between both mediators (21). Grb10-induced Akt activation was observed in the absence of increased PI 3-kinase activity suggesting a Grb10 regulatory mechanism downstream of PI 3-kinase possibly as a result of the direct association with Akt (21). Consequently, Grb10 is well-positioned to regulate the PI 3-kinase pathway at multiple levels and may define the specificity of the signal output along the various branches of the metabolic insulin signal.

5.3. Regulation of the balance between p85 and p110 PI 3-kinase defines the polarity of the signal

An additional role of Grb10 may lie in the regulation of the p85-p110 PI 3-kinase complex. The exact stoichiometry between cellular p85 and p110 is critical to define the resulting PI 3-kinase signal (55, 56). Depending on this balance elevation of p85 or p110 levels will either increase or decrease the resulting signal depending whether this elevation will increase or decrease the number of active p85-p110 complexes in a specific cellular background. Thus to classify p85 as a positive or negative regulator of the PI 3-kinase signal is meaningless and will result in contradictory definitions unless the underlying cellular background, such as the balance between p85 and p110 is taken into consideration (55, 56). The observed experimental differences in response to elevated Grb10 levels may be similarly explained by the resulting balance with other key signaling mediators, including the p85-p110 PI 3-kinase complex which plays a major role as a Grb10 target (22). In this context elevation of cellular Grb10 levels would either stimulate or inhibit the PI3-kinase signal depending on the exact cellular context. Inhibitory effects have been typically observed at high expression levels such as in IR over-expression systems including CHO cells or after lytic infection of mouse 3T3-L1 adipocytes with recombinant adenovirus at Grb10 expression levels up to 20-fold over basal levels. Under these conditions Grb10 may reach a level of excess over relevant cellular partners that will actually reduce the regulated signal such as frequently measured at the level of molecular mechanisms *in vitro* centering around PI 3-kinase (5, 37, 46). On the other hand, increased cellular Grb10 through the introduction of cell membrane-permeable peptides into mouse 3T3-L1 adipocytes has been found stimulatory on all tested metabolic insulin responses and many mediators. Accordingly, dominant-negative approaches using Grb10 domain-specific peptide mimetics interfered with the respective mechanisms. Other signaling mediators and pathways that were not affected demonstrated the specificity of the Grb10 response (22). Based on the direct association between Grb10 and p85, competition between p110 and Grb10 is conceivable for association with p85. A similar concept has been proposed for Grb10 at the level of IR to block access of IRS-1/2 to IR (46).

5.4. Putative role in cell survival through direct interaction with Raf1 and MEK

Human Grb10 zeta interacts via its SH2 domain with MEK1, but not with the closely-related MEK4 (SEK1/JNKK/MKK4) in co-immunoprecipitation and *in vitro* binding experiments (20). Grb10 and MEK1 associate with Raf1 *in vitro*. This association was independent of tyrosine phosphorylation but was inhibited by treatment with alkaline phosphatase. Consequently, either Grb10 or its interacting partners Raf-1 or MEK1 need to be phosphorylated on serine/threonine to enable the interaction. Raf1-Grb10 association was found to be constitutive whereas binding of MEK1 to Grb10 was peptide hormone-dependent. A reversible apoptotic phenotype was induced by over-expression of complete Grb10 carrying point mutations in the SH2 domain (which disrupt association with MEK1 and/or receptor tyrosine kinases) and this phenotype was rescued by co-expression of complete Grb10. Mutations in the Grb10 SH2 domain may sequester signaling components that are normally required for cell survival (57). Peptide hormone stimulation recruits Grb10 to the activated receptor at the cell surface. In COS-1 and HeLa cells cellular Grb10 was found in mitochondria while over-expressed Grb10 accumulated in the cytoplasm. Serum or IGF-1 stimulation induced transient translocation of a small endogenous Grb10 fraction to the membrane, consistent with the formation of receptor tyrosine kinase signaling complexes. Cell-fractionation combined with co-immunoprecipitation confirmed the mitochondrial co-localization of cellular Grb10, Raf1, and MEK1 (57). Grb10 and Raf-1 association was elevated in mitochondrial extracts of UV-treated, apoptotic cells and Grb10 has been proposed to regulate programmed cell death by modulating the anti-apoptotic properties of mitochondrial Raf-1 (57). Grb10 may serve as a link between cell surface receptors and the apoptotic machinery in the outer mitochondrial membrane, presumably by interacting with the PI 3-kinase/Akt signaling pathway.

5.5. Grb10 gene disruption and the physiologic role of Grb10 in the context of cross-regulation within the super family

It is well established that Grb10 is subject to genomic imprinting with the majority of Grb10 expression arising from the maternally inherited allele in the mouse embryo whereas the paternal allele is responsible for the majority of Grb10 expression in the adult mouse (58). The timing and mechanism of the underlying switch remain to be elucidated. Disruption of the maternal allele causes overgrowth of embryo and placenta resulting in mice of a 30% increased birth weight (43). This phenotype can be explained by two alternative major concepts. Loss of Grb10 may remove a mechanism that normally constrains embryonal growth such as a putatively inhibitory function of Grb10. Alternatively, loss of Grb10 may change the balance of related signaling mechanisms possibly involving Grb7 and Grb14 in a way that results in a net up-regulation of embryonal growth. The first interpretation would suggest a negative mitogenic role of Grb10 in mouse embryogenesis that is compatible with other observations in adult tissues (16, 37, 46). On the other hand, the latter interpretation is strongly supported by the observation that increased levels of Grb10 stimulate mitogenesis and cell proliferation in cultured fibroblasts whereas peptide mimetics of individual Grb10 domains inhibit the same

responses (15, 24; Deng and Riedel, unpublished data). Presumably, Grb10 function is connected to the balance of associated signaling mediators including PI 3-kainse (see above) that will differ in alternative host cells and affect the polarity of the output signal. Grb10 disruption in the mouse did not yet shed light on the role of Grb10 in metabolic insulin action except for a high glycogen content that was observed in liver hepatocytes and remains to be interpreted (43). In this context, Grb10 action was found essentially independent of insulin-like growth factor-2 indicating that imprinting acts on at least two major fetal growth axes and suggesting against a role of IGF-IR in Grb10 growth regulation. This interpretation is not supported by the interaction of Grb10 with ubiquitin ligase Nedd4 and the resulting ubiquitination and subsequent degradation of IGF-IR (36). The liver and placental hyperplasia caused by Grb10 gene disruption have been interpreted to suggest a possible connection to c-Met signaling (43) since gene disruption of its ligand, hepatocyte growth factor results in a reduction in liver size and placental defects (59). An association between a Grb10 SH2 domain peptide mimetic and activated c-Met has been demonstrated *in vitro* (15). The context of the super family opens the possibility of functional overlap between Grb10, Grb7, and Grb14. This is supported by functional redundancy in the modulation of mitogenic, metabolic, and chemotactic mechanisms as well as by some complementarity in the tissue distribution in particular between Grb7 and the other super family members.

5.6. Cross-talk to Grb7 and Grb14 signaling and its impact on the interpretation of Grb10 function

Grb10 gene disruption may potentially up-regulate Grb7 or Grb14, the other members of the super family which may stimulate growth consistent with some of their reported cellular roles (see adjacent reviews 60, 61). This could alternatively explain the observed phenotype in the absence of a growth inhibitory role of Grb10 that has not yet been addressed (43). The disruption of the Grb10 gene may also change the balance between Grb7 or Grb14 and Grb10 in a putative competition for shared cellular targets. Functional overlap within the super family is suggested by the shared domain structure and by experimental observations about Grb7 and Grb14 that have been summarized below starting with Grb7 which associates with a number of receptor and non-receptor tyrosine kinases (32, 62). Aspects of Grb7 function such as its enhanced expression in certain breast, gastric, and esophageal cancer cell lines including co-amplification with HER2/erbB2 are consistent with a stimulatory mitogenic role that remains to be established (63, 64, 65, 66, 67). Grb7 associates with a number of mediators with an implicated role in cell motility including erbB2, focal adhesion kinase (FAK), and Tek/Tie2 (68). Grb7 is targeted to focal contacts and phosphorylated by focal adhesion kinase in the regulation of cell migration in contrast to Grb10 and Grb14 for which a role in cell motility has not been shown (69, 70). Grb7 stimulates integrin-mediated cell migration whereas its SH2 domain interferes with this response and appears to act dominant-negatively (69, 70). The SH2 domain does not interfere with cell cycle progression (71), however, Grb7 stimulates anchorage-independent growth via binding to caveolin (72). Broad functional overlap has also been described for Grb14. The reported over-expression of Grb14 in a subset of human breast and prostate cancer cell lines would be compatible with a stimulatory mitogenic role of Grb14 which remains to be demonstrated (44). Grb14 may play a role in PDGF signaling as a substrate of a PDGF-regulated serine kinase (44) and associates with activated fibroblast growth factor (FGF) receptor compatible with a role in FGF signaling (73). A mutation in the SH2 domain renders Grb14 unable to associate with FGFR and potentiates the effect of FGF whereas Grb14 over-expression inhibits FGF-induced proliferation (73). Grb14 over-expression decreases the insulin-mediated phosphorylation of IRS-1 and other IR targets, interferes with glycogen and DNA synthesis (39, 74) but leads to sustained IR phosphorylation (75). Consequently, the evaluation of the consequences of Grb10 gene disruption will remain incomplete in the absence of any information on its impact on Grb7 and Grb14 signaling.

The high level of amino acid sequence similarity between super family members complicates the interpretation of experimental findings due to likely cross-talk under conditions where multiple members are expressed. High amino acid similarity extends throughout much of the proteins except for the N-terminus where it is limited to one Pro-rich sequence. For the SH2 domain, the most highly conserved region, amino acid identity reaches up to 74% for Grb14 and up to 67% for Grb7 when compared to Grb10 sequences (44). As a result over-expression of Grb10 is likely to cross-regulate Grb14 and/or Grb7 signaling, in particular, at high expression levels where Grb10 may out-compete cellular Grb14 or Grb7 for access to their normal targets. In the context of insulin action such cross-talk is expected to result in an inhibitory signal since, overall, an inhibitory role of Grb14 has been implicated in insulin action based on the available data as reviewed above (39, 74, 75). Despite its reported association with activated IR, a role of Grb7 in insulin action has not been suggested (62). The observations suggesting an inhibitory role of Grb10 in insulin action should consequently be interpreted with caution (5, 37, 46, 47) until putative cross-talk to the Grb14 signaling mechanisms has been addressed. Experimental conditions have been established which indicate a stimulatory role of Grb10 in metabolic insulin action based on multiple lines of evidence (22, 33). While the employed reagents such as mouse Grb10 delta cDNA and the SH2 domain or Pro-rich peptide mimetics clearly share high sequence similarity to Grb14 and Grb7 sequences (44), the experimental results and conclusions are clearly incompatible with a putative cross-talk to Grb14 signaling mechanisms (22). At the mitogenic level an inhibitory role of Grb14 has been suggested in insulin and in FGF signaling (39, 73) whereas Grb7 is co-amplified in various cancers with an implicated stimulatory role in cell migration and invasion (76). The interpretation of an inhibitory mitogenic role that has been suggested for Grb10 in IGF-I action could possibly be explained by cross-talk to Grb14 signaling although a rationale has been provided by the role of Grb10 in IGF-IR degradation through its interaction with ubiquitin ligase

Nedd4 (16, 35, 36). The stimulatory mitogenic role that has been described for Grb10 in response to various peptide hormones is not easily explained by cross-talk to Grb14 signaling since this should result in an opposite, inhibitory outcome. An explanation of the results through cross-talk to Grb7 signaling is unlikely since Grb10 stimulation was found to be pathway-specific for the insulin, IGF-I, and PDGF responses but excluding the EGF response (15). Cross-talk to Grb7 signaling should result in an EGF response since a role of Grb7 has been implicated in EGF action (67, 77) whereas Grb10 does not appear to participate (15).

5.7. Grb10 in embryonic development and in the central nervous system

Grb10 is ubiquitously expressed in the mouse embryo including placenta and has been implicated in embryonal growth defects (29) and in the development of the enteric endocrine and renal systems (3). Disruption of the maternal allele of Grb10 in mouse causes overgrowth of embryo and placenta (43) as discussed above. The Grb10 gene was also identified as one out of seven maternally imprinted candidate genes in mouse embryogenesis (29). A key role has been proposed in imprinting effects of prenatal growth retardation or embryonal growth promotion caused by maternal (MatDp.prox11) or paternal duplications (PatDp.prox11) of proximal chromososme 11 with reciprocal deficiencies (78). A potential role of Grb10 in Silver-Russell Syndrome, characterized by intrauterine growth retardation with a wide variety and degree of documented traits, but notably excluding mental retardation (http://magicfoundation.org/rss.html), is discussed in the adjacent review (1). Studies of 139 patients failed to detect mutations in the Grb10 protein-coding region, however, possible epigenetic alterations or mutations that regulate Grb10 expression remain to be investigated (31, 79). Intriguingly, genes have been discovered in this chromosomal region that use intron and antisense sequences of overlapping genes (80), suggesting a potentially complex regulation of Grb10 expression. Since Grb10 is embedded in a heavily imprinted gene cluster, long-range effects on the distally located gene *COBL* (see below) should be considered as well (31). A role of *COBL* has been suggested in axional patterning (81). In addition, a role of Grb10 in neural signaling is suggested by its abundant expression in mouse brain (2) as shown in Figure 2 and by its association with the Eph-related receptor tyrosine kinase ELK which implicates a role in axonal guidance and neuronal bundling (13). Based on SNP expression analysis in mouse strains with uniparental disomy of chromosome 11, the Grb10 gene appears to be biallelically expressed in the brain (31). In contrast, Grb10 expression is ubiquitous during embryogenesis including placenta and resticted to the maternal allele (29) whereas expression is limited to specific tissues during adulthood (Figure 2) and restricted to the paternal allele (2, 58). Mouse Grb10 is located on chromososme 11 between the centromere and G-band 11a3.2 (30) and is flanked in both human (chromososme 7) and mouse (chromosome 11) by the genes *DDC* (dopa-decarboxylase gene) proximally and *COBL* (*Cordon-Bleu* gene) distally. *DDC* is involved in the synthesis of neurotransmitters dopamine and serotonine that are important in brain function. Due to its proximity with the imprinted Grb10, mutual long-range effects on the regulation of gene expression should also be considered for *DDC* (31). One effect of *DDC* de-regulation is exemplified in Parkinson's disease. This neuro-degenerative disorder is characterized by initial dopamine deficiency with decreased levels of dopa-decarboxylase as the disease progresses (82). Angelman syndrome, a genetic disorder characterized by mental retardation, seizures, tremor, ataxia, and other traits has been linked to loss of function mutations of E6-associated protein (E6-AP) ubiquitin protein ligase (UBE3A) which suggests a putative role of ubiqitin-mediated protein degradation during brain development (83, 84). In the mouse, major phenotypic features of Angelman syndrome correlate with loss of expression of UBE3A in hippocampus and cerebellum (85). Grb10 has been found to associate with a member of the ubiquitin-ligase (E3) family, Nedd4-1 (*n*euronal precursor cell-*e*xpressed *d*evelopmentally *d*ownregulated 4) to down-regulate IGF-IR in mouse cells (16) compatible with a possible role of Grb10 in brain development and function.

First direct evidence has been reported for a role of Grb10 in rat brain function. Grb10 is highly expressed in the olfactory bulb together with the voltage-gated potassium channel Kv1.3 of the *Shaker* family (86). The biophysical properties of Kv channels are regulated by conformational changes induced by phosphorylation and possibly by protein-protein interactions with intracellular partners (87, 88, 89). A role of Grb10 has been implicated in the modulation of v-Src-induced Kv1.3 channel function in over-expressing cell lines. Grb10 over-expression significantly reduced v-Src-induced Kv1.3 tyrosine phosphorylation, current suppression, and disrupted cumulative inactivation properties. However, Grb10 appears to modulate Kv1.3 activity also in a phosphorylation-independent manner since co-expression with a kinase-impaired v-Src mutant (R385A) resulted in altered current magnitude and inactivation kinetics. Other effects of v-Src on Kv1.3 function, like the shift in voltage dependence, were not altered by Grb10 over-expression. The following model of Grb10 action on potassium channel function has been proposed (86): Kv1.3 and Grb10 proline-rich regions may compete for interaction with the Src SH3 domain such that the Src SH3 domain preferentially binds to a Grb10 proline-rich target. Grb10 may regulate v-Src modulation of Kv1.3 by blocking the access of the kinase to the ion channel and thereby 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. Overall, Grb10 action to modulate the v-Src kinase-activated Kv1.3 potassium channel appears to be complex and may involve multiple mechanisms and sites of interaction (86). Future studies should address whether Kv channel function is indeed regulated by Grb10 in olfactory bulb cells.

5.8. SH2 domain provides an intra-molecular interface for Grb10 dimerization

The Src-homology 2 (SH2) domain represents a protein module of approximately 100 amino acids (Figure

1) that binds to phosphotyrosine residues with specificity and selectivity defined by 3-6 additional C-terminal amino acid residues (90). The crystal structure of the human Grb10 gamma SH2 domain revealed that unlike typical monomeric SH2 domains the Grb10 SH2 domain is dimeric in solution in combination with data from gel filtration and sedimentation experiments (26). The structure of the Grb10 SH2 domain is represented by a core anti-parallel beta sheet flanked on both sides by an alpha helix and broadly resembles other SH2 domains. In particular, alpha-helix B in the C-terminal half of the SH2 domain contributes to the dimer interface. Sequences involved in the dimer interface are strictly conserved among the Grb7/10/14 super family and suggest a consistent dimeric structure with the exception of Phe 496 which is replaced by tyrosine in Grb7 (26). Located at a key position in the center of the dimer interface, replacement of Phe 515 by Arg resulted in SH2 domain monomers as detected in gel filtration and sedimentation experiments. Complete human Grb10 gamma forms oligomers and resulting either in an elongated dimer or in a tetramer (26). In a typical SH2 domain-phosphopeptide association, the phosphotyrosines in the +1 and +3 positions of the phosphopeptide fit into two deep pockets in the SH2 domain. Since the binding pocket for the amino acid in +3 is absent in the Grb10 SH2 domain, dimerization should favor binding of dimeric, turn-containing phosphotyrosine sequences, such as the activation loop of the two beta subunits of IR and IGF-IR.

5.9. Grb10 dimerization and multi-domain structure provide interfaces for the assembly of signaling complexes

The multi-domain structure of Grb10 opens the potential for a role as a signaling hub to integrate parallel signals or to provide cross-talk between pathways as well as serving as a scaffold to organize signaling complexes (Figure 4). An intriguing possibility lies in the connection of parallel signaling pathways. A putative role of Grb10 as a link between IGF-I and PDGF action at the level of the receptor will serve as an example (33). The interaction of Grb10 with either receptor is mediated by distinct domains, the BPS domain in the association with IGF-IR versus the SH2 domain in the association with PDGFR beta (15, 25). Consequently, Grb10 may simultaneously interact with either receptor and functionally link both pathways. A variety of signaling mediators may simultaneously bind to the repeated Pro-rich, RA-like, PH, BPS, SH2, and to putative, additional interfaces of Grb10 that remain to be defined. The dimerization of Grb10 (26) enhances this concept and may essentially double the number of signaling mediators that can assemble into specific complexes through association with Grb10 binding interfaces.

5.10. Phosphorylation of Grb10 sequence motifs may provide a key regulatory mechanism of interactions with signaling partners

The reported phosphorylation of Grb10 in response to many peptide hormones (2, 11, 19, 91) in combination with multiple, putative kinase target sites (Figure 1B) provides the opportunities for multiple interactions and regulatory mechanisms. Basal phosphorylation on serine has been reported for mouse Grb10 that was stimulated in response to EGF; similarly PDGF and fibroblast growth factor caused a mobility shift in the migration of Grb10 on SDS gels that was reversible by phosphatase treatment (2). Basal serine phosphorylation of human Grb10 zeta was also stimulated by insulin, which was reversible by phosphatase, the MEK1 inhibitor PD98059, or the PI 3-kinase inhibitor wortmannin (11). Tyrosine phosphorylation of Grb10 has been reported in response to insulin or vanadate that involves Src family tyrosine kinases such as Src and Fyn (7, 91). Grb10 has been described as a direct substrate of the Tec tyrosine kinase indicating a role of tyrosine phosphorylation in Grb10 function and providing a link to cytokine action in the hematopoietic system (18, 19).

5.11. PH region defines variant-specific inter- and intra-molecular interactions

Members of the Grb7/10/14 super family share a pleckstrin homology (PH) region (Figure 1), originally identified in the cytoskeletal protein pleckstrin (92, 93) and found in diverse proteins involved in cellular signaling, cytoskeletal organization, and regulation of membrane trafficking (94, 95). PH domains as shown for Grb7 within the super family (96) bind phosphinositides that are routinely produced in response to activation of cell surface receptors and result in protein translocation to the plasma membrane (97). Human Grb10 beta carries a variant-specific and incomplete PH region that does not prevent insulin-stimulated membrane translocation (10). Since PH regions also mediate protein-protein interactions, an intact PH region may support Grb10 association via the BPS and SH2 domains with IR and IGF-IR (10, 25). The PH region also participates with the SH2 and BPS domains in Grb10 oligomerization based on two-hybrid interaction studies (41). This implies a role in intra-molecular as well as in intermolecular interactions that remains to be elucidated.

6. FUTURE PERSPECTIVE

6.1. Cell membrane-permeable peptides as versatile probes to study Grb10 domain function

An important strategy is represented by Grb10 gene disruption experiments in the mouse that have been reported but are complicated by the observed genetic imprinting (43). A more comprehensive study should provide further insight into the cellular role of Grb10 but will need to consider Grb7 and Grb14 signaling that may be altered by Grb10 gene disruption and may result in cross-talk. As one example, expression of the Grb10 gene itself is up-regulated by vascular endothelial growth factor in a putative positive feedback signaling loop (98). Mice carrying gene disruptions in any two super family members can be interbred to test the impact of varying gene doses for each protein and the resulting potential cross-regulation of signaling mechanisms. Opposing phenotypes may be caused by complex parameters (99) such as the acute/phasic or chronic nature of a participating signal as shown for the MAP kinase cascade (100). Cell membrane-permeable peptide mimetics provide an alternative tool to directly interfere with the function of an individual Grb10 motif in a domain-, mediator-, pathway-specific, and dose-dependent manner (15, 22). Reported results were

indistinguishable from cDNA expression approaches in a direct comparison with the advantage that cell membrane-permeable peptides are delivered more effectively than cDNA into any cell including terminally differentiated target cells. In addition, cell membrane permeable peptides enter cells and act within minutes and have been detected up to one week after delivery, surpassing the operational time window provided by transient cDNA expression systems (101, 102). Domain-specific peptide mimetics allow specific interference with individual functional motifs of cellular Grb10 with excellent signal-to-noise ratio due to the effective delivery into all cells. This approach is in many ways comparable to RNA interference strategies with the advantage that rather than being limited to a complete transcript the function of specific proteins domains is directly and individually disrupted. Delivery of cell membrane-permeable peptides into the mouse has been reported reaching most tissues and crossing the blood-brain barrier (103). This provides a rapid approach to test the function of individual Grb10 domains in an intact animal as long as a phenotype is observed over a defined time period in an adult mouse. Disadvantages lie in the need for repeated peptide delivery to each individual animal including the lack of genetic transmission to offspring and in the ubiquitous peptide distribution throughout the organism that cannot be limited in a tissue-specific fashion.

6.2. Identification of Grb10 binding partners by genetic, biochemical, functional genomic, and imaging strategies

An effective way to elucidate the function of Grb10 lies in the identification of its cellular signaling partners (Figure 4). Historically, the yeast two-hybrid system has been instrumental in identifying Grb10 itself and many of its signaling partners. It remains a viable future strategy to identify associated mediators, in particular, from tissue-specific libraries and if combined with a protein kinase to allow the screening of phospho-protein interactions. On the other hand with the increasing definition of the proteome it may become more effective to identify interacting partners biochemically via co-immunoprecipitation with Grb10 in a defined cellular context and based on their specific molecular weight that can be matched with the existing knowledge base of signaling mediators. If this approach remains inconclusive, associated proteins can be frequently identified by peptide mass fingerprinting. This approach may soon be complemented by comprehensive proteome micro arrays that will help define Grb10 binding partners directly based on protein-protein interactions. In the immediate future it will be compelling to exploit the rapid advances in the development of genome-wide DNA arrays for a comprehensive survey of changes in gene expression in response to either increased levels of specific Grb10 variants or to interference with specific Grb10 domains in a chosen cellular background. At the cellular level it will be critical to increase the resolution of the experimental analysis with respect to the duration and timing of signals and to the local distribution of the involved signaling mediators including their sub-cellular localization. Signaling specificity requires the spatially controlled assembly of complexes, aided by scaffolding functions which may be contributed by Grb10 as well as spatial compartmentalization by cellular organelles (104). The detailed analysis of the temporal and spatial organization of Grb10 such as by confocal or two-photon fluorescence microscopy should provide insight into functional aspects. This is exemplified by the reported mitochondrial localization of Grb10 that implicates a role in the regulation of cell survival (57) as well as by the localization of Grb7 to focal adhesions that helped elucidate its role in integrin-mediated cell migration including tumor progression (76). At the moment the best available evidence points to a role of Grb10 in peptide hormone action in particular in response to insulin and IGF-I. Compatible with its multi-domain structure Grb10 may act as a signaling scaffold or as a hub to integrate signals from alternative pathways. Signaling nods in protein interaction networks have been proposed to connect functional protein groups in this context. Despite the substantial effort that has been invested in the analysis of the underlying signaling pathways the critical participation of Grb10 had been overlooked for many years. With the progressing elucidation of its molecular roles Grb10 may emerge as a new focal point in cellular signaling and provide a target for future therapeutic intervention.

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Abbreviations: BPS: between PH and SH2; MAPK: mitogen activated kinase; IGF-I: insulin-like growth factor I; IR: insulin receptor; IGF-IR: IGF- I receptor; EGF: epidermal growth factor; Grb: growth factor receptor binding protein; PDGF: platelet-derived growth factor; PH: pleckstrin homology; SH2: Src homology-2

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